Lipopolysaccharide of Different Bacteria: Extraction methods, Signalling and Cytokine Production.

Mohammad Abdullah Mubarak Al-Hawi

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Contents

Dedication

If ever they pull this off the shelf to browse, I dedicate this thesis to them:

My parents, They bore me, raised me, supported me, taught me, loved me and they are the main reason why I am here right now.

My wife, Razan, words cannot express my deepest gratitude and love. Supporting me for so long has never gone unnoticed.

My children, Afnan, Abdullah and Suhail, whose enriching my life in ways I could never describe.

Abbreviations

Act	Actinomycin D
AP	Aqueous Phenol
BCR	B cell receptor
BF1p	Purified *Bacteroides fragilis* extracted by TM
BF1u	Unpurified *B. fragilis* extracted by TM
BF2p	Purified *B. fragilis* extracted by TMP
BF2u	Unpurified *B. fragilis* extracted by TMP
BF3p	Purified *B. fragilis* extracted by PCP
BF3u	Unpurified *B. fragilis* extracted by PCP
BF4p	Purified *B. fragilis* extracted by BWP
BF4u	Unpurified B. f *B. fragilis* extracted by BWP
BF5p	Purified *B. fragilis* extracted by AP
BF5u	Unpurified *B. fragilis* extracted by AP
BPI	Bactericidal permeability increasing protein
BWP	Boiling water/ Proteinase K treatment
CETP	Cholesteryl ester transfer protein
CHO	Chinese hamster ovary
DMEM	Dulbeccos modified eagles medium
EC1p	Purified *Escherichia coli* extracted by TMP
EC1u	Unpurified *E. coli* extracted by TMP
EC2p	Purified *E. coli* extracted by TM
EC2u	Unpurified *E. coli* extracted by TM
EC3p	Purified *E. coli* extracted by AP
EC3u	Unpurified *E. coli* extracted by AP
EC4p	Purified *E. coli* extracted by BWP
EC4u	Unpurified *E. coli* extracted by BWP
ECD	Ectodomains
EDL	Electron dense layer
FCS	Foetal calf serum
HDL	High density lipoprotein
HEK	Human embryonic kidney
Hep	L-glycero-D-manno-heptose
HK	Heat killed

HS	Human serum
IL	Interleukin
IRAK	Interleukin- 1 receptor associated kinase
IU	International units
IκB	Inhibitory proteins of NF-κB
Kdo	2-keto-3-deoxyoctonic acid
LAL	Limulus Amoebocyte Lysate
LBP	Lipopolysaccharide binding protein
LC	Large capsule
LDL	Low density lipoprotein
LOS	Lipooligosaccharide
LPS	Lipopolysaccahride
LMW	Low molecular weight
LRR	Leucine rich repeat
LT	Lipid transfer
LTA	Lipoteichoic
MAL	MyD88 adaptor-like protein
mCD14	Membrane CD14
MD-2	Myeloid differentiation protein 2
MOF	Multiple organ failure
MPRL	Microbial Pathogenicity Research Laboratory
MyD88	Myeloid differentiation factor 88
NCTC	National type culture collection
NF-kB	Nuclear factor
PA1p	Purified *Pseudomonas aeruginosa* extracted by TM
PA1u	Unpurified *Ps. aeruginosa* extracted by TM
PA2p	Purified *Ps. aeruginosa* extracted by TMP
PA2u	Unpurified *Ps. aeruginosa* extracted by TMP
PA3p	Purified *Ps. aeruginosa* extracted by AP
PA3u	Unpurified *Ps. aeruginosa* extracted by AP
PA4p	Purified *Ps. aeruginosa* extracted by BWP
PA4u	Unpurified *Ps. aeruginosa* extracted by BWP
PAGE	Polyacrylmide gel electrophoresis
PAMP	Pathogen-associated molecular patterns

PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCP	Phenol/ Chloroform/ petroleum
PFW	Pyrogen free water
PG	*Porphyromonas gingivalis*
PLTP	Phospholipid transfer protein
PPY	Proteose peptone yeast extract broth
PRR	Pattern recognition receptors
RS1	*R. sphaeroides* LPS fully depigmented and extracted by AP.
RS2	*R. sphaeroides* LPS fully depigmented and extracted by PCP.
RS3	*R. sphaeroides* LPS partially depigmented and extracted by PCP.
RS4	*R. sphaeroides* LPS not depigmented and extracted by PCP.
SC	Small capsule
sCD14	Soluble CD14
SDS	Sodium dodecyl sulphate
SIRS	Systemic inflammatory response syndrome
TBS	Tris buffered saline
TCR	T cell receptor
TEA	Triethylamine
TEMED	NNN'N- tetramethyl-1,2- diaminoethane
THP-1	Human acute monocytic leukemia cell line
TIR	Toll/IL-1 receptor
TIRAP	TIR- associated protein
TLR	Toll like receptor
TM	Triton/magnesium chloride
TMP	Triton/magnesium chloride/Proteinase K
TNF	Tumour necrosis factor
TRAM	Trif- related adaptor molecule
TRAF6	TNF receptor activated factor 6
TRIF	TIR related activator of interferon-β
TTBS	Tween tris buffered saline
WCDF	White-count diluting fluid
WSP	Water-saturated phenol

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Abstract

Lipopolysaccharide (LPS: endotoxin) is an essential part of the outer membrane of the Gram-negative cell envelope, and is recognized as one of the potent inducers of immune responses. Endotoxin is also recognised as the main cause of the events leading to septic shock since it is believed that a huge amount of endotoxin crosses the intestinal barrier to reach the blood circulation. Although it is essential to try to identify the interactions between endotoxin and the innate immune mechanisms in particular, it is not possible to isolate these interactions from other possible interactions with other cell wall related structures of protein or polysaccharide origin. This was the reason for the first approach of this study in which different LPS extraction methods were utilized to investigate the differences between them in terms of producing proinflammatory immune response. A further application of a repurification method was to eliminate any possible protein contaminants. These purified LPS preparations were used for the other main approach of this study in which different unpurified and repurified *Bacteroides fragilis* LPSs together with different heat killed *B. fragilis* populations were examined to elucidate their Toll-like receptor (TLR) specificity.

Four different extraction methods were chosen to extract LPS from *Escherichia coli* O18K-, *Pseudomonas aeruginosa* Pa-O1, *Bacteroides fragilis* NCTC 9343 and *Rhodobacter sphaeroides* NCIMB 8253. All of these species, except of *R. sphaeroides*, were able to stimulate the production of proinflammatory cytokines TNF-α and IL-1β with differences apparent between different LPS preparations according to their extraction methods. *R. sphaeroides* LPS was able to inhibit the ability of these LPSs to induce TNF-α production except for *B. fragilis* LPS which was not effected by *R. sphaeroides* LPS. All different *B. fragilis* LPSs showed the ability to exert an antagonist effect on different *E. coli* LPSs on production of TNF-α or IL-1β from both human monocytes and THP-1 cell lines, which indicated that

there was not such a profound effect of the extraction method in totally changing the bioactivity of specific LPS. Moreover, unpurified or purified LPSs of *B. fragilis* on the one hand and heat killed bacteria of *B. fragilis* from different capsular polysaccharide populations on the other hand all showed an obvious TLR2 signalling specificity but not TLR4 specificity. This adds further evidence that different LPS extraction methods with or without applying a repurification procedure do not change the TLR specificity of the *B. fragilis* LPS.

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Declaration

All of the investigations and procedures presented in this thesis were performed by the author unless otherwise indicated in the text.

CHAPTER 1: INTRODUCTION

1.1 General structure of lipopolysaccharide (LPS)

1.1.1 General view

One of main dissimilarities between Gram-positive and Gram-negative bacterial cell walls is that the latter contains an inner and outer membrane. Lipopolysaccharide (LPS) is an essential part of the outer membrane of the Gram-negative cell wall. LPS and endotoxin are terms that describe the same molecule. LPS usually refers to the chemically purified form of the molecule whereas it is called endotoxin when it is found inside the host (Radon, 2006). The discovery of endotoxin by Richard Pfeiffer in 1892, as a determinable bacterial structure, paved the way to understand how microbes create disease (Beutler and Rietschel, 2003). According to current knowledge, lipopolysaccharide is considered to be a fundamental structure for the viability of all Gram-negative bacteria and one of the most powerful microbial inducers of inflammatory immune responses (Alexander and Rietschel, 2001; Dobrovolskaia and Vogel, 2002). However, two exceptions to this rule have been observed. Firstly, some bacteria contain glycosphingolipids rather than LPS. Secondly, an LPS-deficient mutant of *Neisseria meningitidis* was described that has the required viability for survival (Gronow and Brade, 2001).

Since the 1950s, techniques of extracting and purifying lipopolysaccharide have been developed. These have been used to verify its basic structure by adapting and applying methods linked to sugar and lipid chemistry (Caroff *et al.,* 2002). In this regard, it is known that each single bacterial cell contains about 3.5×10^6 molecules of LPS (Rietschel *et al.,* 1994) and every LPS molecule has a molecular weight that varies between 2000 to 20,000 (Caroff *et al.*, 2002). 75% of the bacterial surface area is assumed to be occupied by LPS molecules which represented about 10-15% of the total molecules in the outer membrane (Lerouge and Vanderleyden, 2002). The lipopolysaccharide structure is conceptually heat-stable, non-proteinaceous and consists of lipid and carbohydrate (Erridge *et al.*, 2002; Dixon and Darveau, 2005). Having carbohydrate linked to a lipid is a general method for firmly attaching a hydrophilic biopolymer to the outer surface of a membrane (Wilkinson, 1996). As a general guideline, LPS represents a particular family of polymers which (a) are part of the outer membranes of Gram-negative bacteria, (b) contain a distinctive

phospholipid anchor "Lipid A", (c) are built according to a common and principle design and (d) possess specific biological properties in term of endotoxicity and O antigenicity (Wilkinson, 1996).

With regard to their general structural architecture, all forms of LPS are comparable (Heumann and Roger, 2002). Structurally, most types of LPS are composed of three characteristic segments: (a) a conserved hydrophobic Lipid A moiety embedded in the membrane, (b) the core oligosaccharide, and (c) the O-specific polysaccharide chain which is called O-antigen (Hurley, 1995a; Wilkinson, 1996; Caroff *et al.*, 2002; Dixon and Darveau, 2005). In a manner consistent with the general LPS architecture, there is a huge diversity of natural structural alternatives that are mainly due to an enormous variety in the chemical composition of the polysaccharide region, followed by the less diverse core region and finally the reasonably conserved Lipid A region (Alexander and Rietschel, 2001; Dobrovolskaia and Vogel, 2002). Conserved domains of LPS are common in bacterial species and contain essential components which maintain either the integrity and/or preserve the whole structure and survival of the bacterium itself. The variable domains represent those parts which are not critical for the bacterium and lacking them does not lead to devastating consequences. In fact, lacking of such variable domains may also have an advantageous nature for the microbe itself.

Generally, alterations in the length of the segment within these domains can result in simple variations of LPS. On the other hand, changing the overall chemical construction or attached charge groups can have remarkable effects which, in turn, can damage the overall structure (Dixon and Darveau, 2005). Nevertheless, typical LPS containing all three segments is found in *Escherichia coli* and other bacterial species related to enterobacteria and is often called smooth or S-form chemotype of LPS (Morrison and Leive, 1975; Poxton, 1995; Wilkinson, 1996; Amor *et al.*, 2000; Beutler and Rietschel, 2003). On the contrary, the genus of *Sphingomonas* is so far the only group of Gram-negative wild-type bacteria which do not express LPS. Instead, the outer membranes of these microorganisms have been shown to contain glycosphingolipids (Kawahara *et al.,* 1991).

1.1.2 O polysaccharide

Isolates of the families Enterobacteriaceae, Pseudomonadaceae, Pasteurellaceae and Vibrionaceae, as well as many other Gram-negative bacteria form a smooth LPS molecules (S-form) in which O-specific chain consists of a maximum of 50 repeating oligosaccharide entities composed of 2–8 monosaccharide moieties in a highly specific style (Alexander and Rietschel, 2001; Raetz and Whitfield, 2002). Bacterial species from the Enterobacteria can synthesize O-specific domain since they have the responsible gene cluster which is called *wb* or *rfb*. So it is unequivocal to extrapolate that bacterial mutants having either defect in, or loss of, this locus synthesize O-polysaccharide deficient LPS which is historically known as rough (R-form) chemotype or deep rough mutants (Rietschel *et al.*, 1994; Poxton, 1995; Alexander and Rietschel, 2001).

The terms O-specific polysaccharide and O-antigen are often interchangeable in view of the fact that the O-polysaccharide is the outermost domain of LPS thus it is a very exposed antigen targeted by highly specific host antibody response (Erridge *et al.*, 2002).

It is worth mentioning the general functionality of the O-polysaccharide domains of LPS since it gives a protective role against host antibacterial defences such as bile acids and cationic peptides or Lipid A recognition receptor (Alexander and Rietschel, 2001).

Generally, the O-specific chain is characterized by a remarkably high structure changeability in term of nature, ring form, sequence, substitution, and type of linkage of constituent monosaccharides such as acetyl, ketal, and glycosyl residues (Rietschel *et al.*, 1994; Keenleyside and Whitfield, 1996; Caroff *et al.*, 2002; Erridge *et al.*, 2002). In addition to that, such heterogeneity is observed even within a specified bacterial species, most plausibly because of the same bacteria synthesize different LPS molecules in term of the length of O-specific chains. In line with the practicality of this, S-form LPS can be detected as a "ladder pattern" of repeatedly spaced bands when examined by polyacrylamide gel electrophoresis and stained with silver (Poxton, 1995; Wilkinson, 1996).

This heterogeneity provided the chemical basis for the serological specificity of a particular wild-type bacterial strains based on the surface O-antigenic properties (Rietschel *et al.*, 1994; Alexander and Rietschel, 2001). For example, there are more than 170 known O-antigens of *E. coli* (Amor *et al.,* 2000). Moreover, it is documented that the O antigen variations between *E.coli* O1, O7 and O18 are related to differences in the nature of pathogenicity (Pluschke *et al.*, 1983b; Achtman and Pluschke, 1986).

On the other hand, the in-vitro capability of rough mutants to survive and reproduce indicate that principally the O-chain is not essential for bacterial viability. However, in tissues or body fluids, many pathogenic Gram-negative bacteria can only survive by virtue of an O-specific chain which in in-vivo situations protects the bacteria from phagocytosis and obstruct the access of serum complement to the Lipid A (Pluschke *et al.*, 1983a; Alexander and Rietschel, 2001; Dobrovolskaia and Vogel, 2002; Fernandez-Prada *et al.*, 2003). Intriguingly, once the reaction between O chain and complement occurs far away from the bacterial cell surface, complement is unable to release its effect. By extrapolation, it could be assumed that the long O antigen has the potential to stop host complement from exerting its ability to lyse bacterial cells. In comparison with this situation, a short or removed O chain allow direct antibody-antigen reaction which lead to bacterial lysis (Lerouge and Vanderleyden, 2002).

Another interesting observation obtained from the phenomenon of molecular mimicry between O antigen from *Helicobacter pylori* and host antigen in which both molecules have a close chemical relationship. This in turn, does not allow immune cell to differentiate between them and as a result an immune response cannot occur (Appelmelk *et al.,* 2000). However, many studies reported LPS of wild-type species of pathogenic Gram-negative bacteria such as *N. meningitidis, N. gonorrhoeae, Haemophilus influenzae, Bordetella pertussis or Chlamydia trachomatis* as O-specific chain deficient. These species have the specific ability to colonize the mucosal surfaces of the respiratory and urogenital tracts. Furthermore, many of these pathogenic bacteria have been elucidated to express different terminal oligosaccharide domains, which closely resemble human glycosphingolipids.

LPS from these species have also been described as low molecular weight LPS (LMW-LPS) or lipooligosaccharides (LOS) (Preston *et al.*, 1996; Alexander and Rietschel, 2001; Dobrovolskaia and Vogel, 2002).

Nevertheless, the immunogenicity of O-antigen appears to be dependent on the adjuvant effect of the lipid-A component of LPS since it is clear that O-antigen detached from the Lipid A core is not very immunogenic and is often referred to as a hapten (Reeves, 1995).

1.1.3 Core polysaccharide

Compared with the high variability of the O-polysaccharide region of LPS, the heterooligosaccharide core of LPS is relatively more conserved. For example, although there are more than 170 O-antigen for *E. coli*, only five distinctive oligosaccharide cores have been determined (R1, R2, R3, R4 and K12) (Rietschel *et al.*, 1994; Dobrovolskaia and Vogel, 2002).

According to the current state of knowledge, two main regions of the oligosaccharide domain are based on sugar composition (Holst, 1999; Erridge *et al.*, 2002). The relatively more variable outer core which is classically composed of neutral or amino hexose sugars such as D-glucose, D-galactose, D-glucosamine, D-galactosamine or N-acetyl derivatives and the inner core which is attached to Lipid A (Alexander and Rietschel, 2001; Caroff *et al.*, 2002; Erridge *et al.*, 2002; Raetz and Whitfield, 2002). The structure of the inner core is very similar between most Gram-negative bacteria and it contains a L-glycero-D-manno-heptose (Hep) residue and at least one Kdo (2-keto-3-deoxyoctonic acid) moiety (Schletter *et al.*, 1995b; Nikaido, 2003).

Most intriguingly, unlike O-chain and the majority of the core which are dispensable, the Kdo residue is a unique component found in almost all known cores. However, there is a derivative of D-glycero-D-talo-oct-ulopyranosonic acid (Ko) which replaces Kdo in species such as *Acinetobacter haemolyticus* (Raetz and Whitfield, 2002). Kdo is usually considered to be completely vital for the bacterial viability.

Most compelling for this are two observations. The first is that a rough mutant of *H. influenzae* contains the smallest saccharide component of LPS of any bacteria which

has only one Kdo residue attached to the Lipid A (Helander *et al.,* 1988). The second is that *Chlamydia* spp have the smallest core ever known in wild type bacteria which contains only a trisaccharide units of Kdo residue (Rietschel *et al.*, 1994; Dobrovolskaia and Vogel, 2002; Raetz and Whitfield, 2002).

Such observations demonstrate the importance of the Kdo region as a linkage to Lipid A and a diagnostic indicator for LPS. In addition studies have considered producing new antibacterial agents designed to inhibit the Kdo-assembly steps in the synthesis of LPS (Rietschel *et al.,* 1994; Belunis *et al.,* 1995; Wyckoff *et al.,* 1998).

For the whole inner core, one study on hepatocytes from mice strongly suggested the presence of a lectin-like receptor for the LPS inner core region (heptose-Kdo region) on the plasma membrane (Parent, 1990).

1.1.4 Lipid A

Lipid A is the vital structural element of LPS that mediates interaction processes with the innate immune response (Cadenas and Cadenas, 2002). It is an extraordinary glycophospholipid that possesses exceptional structural properties (Poxton, 1995). There had been numerous observations since the early 1950s that pointed towards Lipid A as the "seat of endotoxicity". Clear cut evidence came from the comparison of synthetic Lipid A with "free Lipid A" extracted from the LPS of *E. coli*. These studies concluded that both are equal in term of structure and endotoxic activity (Alexander and Rietschel, 2001; Beutler and Rietschel, 2003).

Hydrophobicity is also a feature that obstructs the complete solubility of Lipid A. Hence, the reduction of the hydrophobic side chains increases solubility of a synthetic lipid compound such as E5564 which is well known for being easily purified and formulated (Rossignol and Lynn, 2002)

At the conceptual level, most bacterial Lipid A molecules have a high level of structure stability which contributes to the almost optimal endotoxic activity. Numerous studies established that Lipid A contains: a) disaccharide residues of (β-1,6-linked D-glucosamine) carrying two phosphoryl groups; a glycosidic (position 1) and a nonglycosidic (position 4'), b) six acyl groups which contain fatty acids of 12

to 14 carbons in length, c) asymmetry of the acylation distribution (4+2) (Rosner *et al.*, 1979; Galanos *et al.*, 1984; Seydel *et al.*, 1984; Tanamoto *et al.*, 1984; Kotani *et al.*, 1985; Erwin and Munford, 1990; Rietschel *et al.*, 1994; Dobrovolskaia and Vogel, 2002; Dixon and Darveau, 2005). Nearly all other LPS molecules which diverge from this structure result in reduced or negligible endotoxic activity. (Galanos *et al.,* 1985; Rietschel *et al.,* 1994)

Accumulated evidences suggested that the receptor-binding specificity of Lipid A is determined by its hydrophilic region (phosphorylated-D-glucosamine disaccharide). Whereas the role in immune cell activation which follows the binding is considered to be controlled by the hydrophobic region of Lipid A (acyl groups) (Rietschel *et al.*, 1994; Alexander and Rietschel, 2001).

The highly conservative level of Lipid A architecture which is comparable between all LPS molecules may lead to conclude that they also have comparable, if not equal, biological actions. The current state of knowledge though clearly indicated that this assumption is far from being a general rule. Instead it is considered that dissimilarity in the Lipid A function is the rule rather than the exception and it closely affects Lipid A activity. These dissimilarities are based on structural domains like the nature of hexosamine found, the extent of phosphorylation, the existence of phosphate substituents and the nature and architecture of acyl groups (Rietschel *et al.,* 1994; Netea *et al.,* 2002).

Examples of LPS structure with high stimulatory activity are those of *E. coli* and many other enterobacterial bacteria such as *Salmonella* spp and *Klebsiella pneumoniae* in addition to nonenterobacterial and enteropathogenic bacteria such as *N. meningitidis* (Gronow and Brade, 2001; Netea *et al.*, 2002; Dehus *et al.*, 2006).

Lipid A isolated from other species varies in terms of the existence of 2,3-diamino-2,3-dideoxy-D-glucose rather than D-glucosamine, the number of acyl groups (4, 5 or 7), chain length, symmetrical allocation (3 + 3 or 2 + 2) or replacement of phosphate groups (Seydel *et al.,* 2000). Many of the penta-acylated Lipid A structures fit in this manner. For example, the LPS of *C. trachomatis, Rhodobacter sphaeroides, Rhodobacter capsulatus* and Lipid A precursor Ia (compound 406) have, in fact, antagonistic actions. Furthermore, some hexa-acylated Lipid A species

like that from *Legionella pneumophila* display low endotoxicity because of its slight modification of an extended acyl groups of at least 18 carbon atoms in length. Another example of low activity Lipid A is the hyperthermophyle *Aquifex pyrophilus* which grows only at high temperatures in the range of 67–95°C. The organism has LPS characterized by a distinctive Lipid A type which entirely lacks any phosphate group and having D-galacturonic acid linked to positions 1 and 4′ as a substitute (Alexander and Rietschel, 2001; Netea *et al.*, 2002). Moreover, another study demonstrated that an intracellular Gram-negative coccobacillus, *Francisella tularensis* has a kind of inert LPS which act neither as agonist to stimulate inflammatory response nor as an antagonist to inhibit other classical LPS (Ancuta *et al.*, 1996).

Much recent attention has focused on the observation that there is a functional relationship between the biochemical composition of Lipid A to its three-dimensional conformation and bioactivity (Seydel *et al.,* 2000). The activity of LPS molecules which have a conical conformation such as that of *E. coli* , is exceedingly high comparing with a cylindrical conformation LPS such as those of precursor Ia (compound 406), *R. capsulatus* or *Chromobacterium violaceum* (Seydel *et al.,* 2000; Netea *et al.,* 2002). Factors like the nature, chain length, number and asymmetry of acyl chains and number and distribution of negative charges, are responsible for such three dimensional conformation of Lipid A (Rietschel *et al.,* 1994; Schromm *et al.,* 1998; Netea *et al.,* 2002). More intriguingly, these characteristics lead some investigators to hypothesize consequently that 'endotoxic activity' is not an exclusive property of endotoxin, but of any other molecule demonstrating such characteristics (Schromm *et al.,* 1999). Figure 1.1 shows the structural characteristics of LPS.

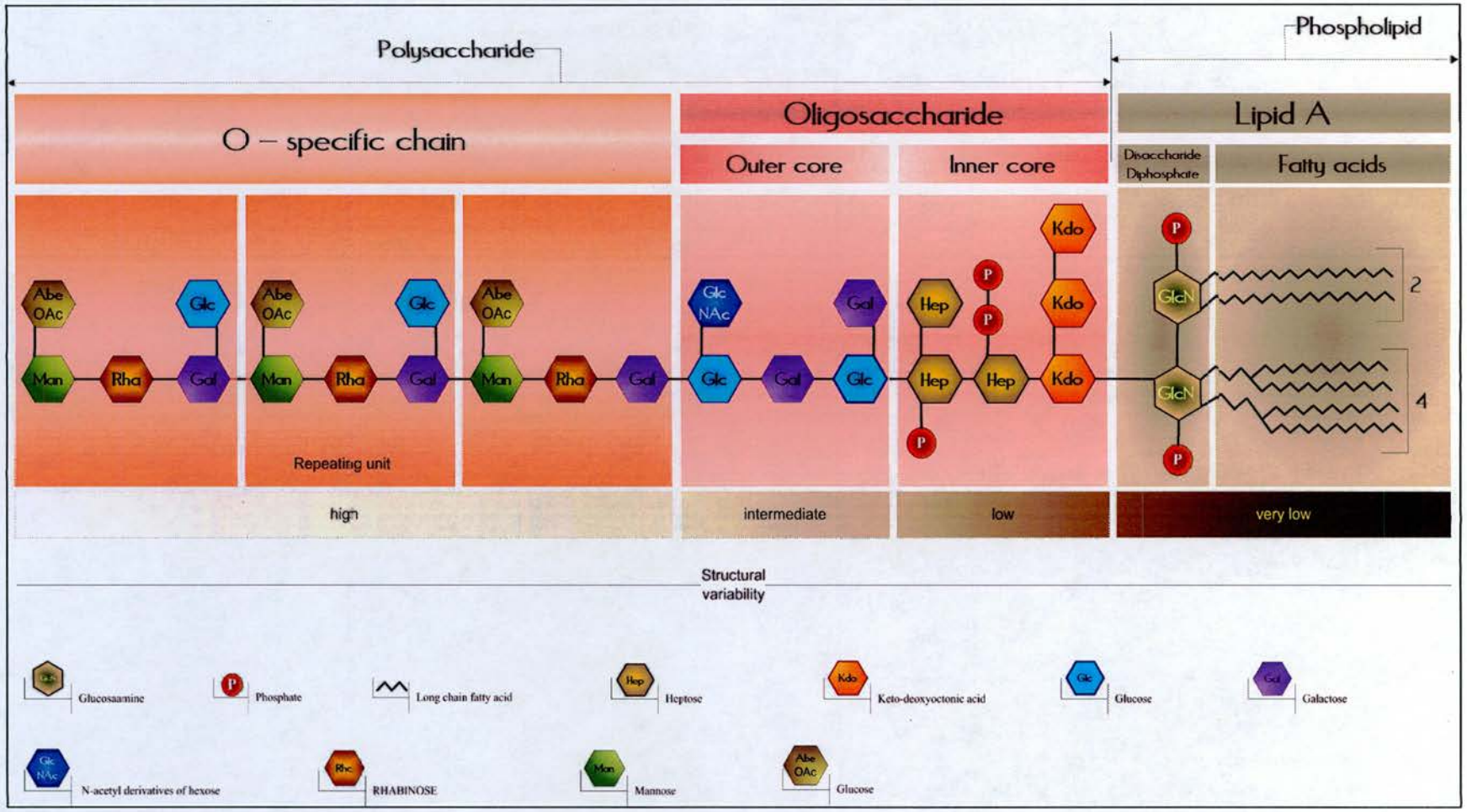

Figure 1.1 Structural characteristics of lipopolysaccharide

1.2 Endotoxin and immune response

1.2.1 General view of innate immunity

The basic functions of the innate immunity are recognizing pathogens, providing an instant obtainable mode of defences without need for previous experience and induction of the adaptive immunity (Vasselon and Detmers, 2002; Kaisho and Akira, 2006). In comparison with adaptive immunity, innate immunity was believed to be rather naive until numerous recent studies prove the high level of complexity of the innate immunity. Endotoxin related studies are considered to be a cornerstone of such studies (Triantafilou and Triantafilou, 2005).

Predominantly, the innate immunity consists of three main defence categories; mechanical, chemical and cellular. The mechanical mechanisms include events like the physical barrier role of the epidermis and mucosa as well as the physiological functions of cilia action, desquamation and mucus discharge. The chemical mechanisms can easily be divided into three subcategories: soluble or cell-linked pattern recognition receptors (PRR), proteins or peptides which have hydrolytic effect on microbes, and cytokines and chemokines that coordinate the immune response. The cellular mechanisms includes many types of host cells such as; epithelial cells, mast cells, dendritic cells, macrophages, granulocytes and natural killer cells (Basset *et al.,* 2003).

In term of recognition machinery of the microbial product, the main dissimilarity between both innate and adaptive immune responses relates to the nature of the receptors. Both T-cell and B-cell receptors of the adaptive immune system are created throughout the development of these cells, in a way that equips each lymphocyte with a structurally unique receptor. These receptors are neither encoded for ever nor are passed on to the next generation. In term of numbers, there are between 10^{14} to 10^{18} different somatically generated T-cell and B-cell receptors (Medzhitov and Janeway, 2000a).

On the other hand, the innate immune receptors are different in term of their presence on a variety of host cell types, crucially on immune cells like macrophages, dendritic cells, most intriguing B cells (the professional antigen-presenting cells) and specific

types of T-cell, and even non-immune cells such as fibroblasts and epithelial cells. (Medzhitov and Janeway, 2000a; Akira *et al.*, 2006). Moreover, these receptors have equal specificities when they are expressed by a particular cell type (e.g., macrophages) although some of which shows various degrees of polymorphism within specific species (Medzhitov and Janeway, 2000a; Vivier and Malissen, 2005). Moreover, these receptors generate an immediate effects rather than waiting for a proliferation process (Medzhitov and Janeway, 2000a).

From the structural point of view, pattern-recognition receptors (PRR) contain many families of protein molecules which include leucine-rich repeat domains, calcium-dependent lectin domains and scavenger-receptor protein domains. In parallel with this, the functional role of PRRs can be achieved by three main modes: secretion, endocytosis and signalling. Mannan-binding lectin is an example of a secreted pattern-recognition molecule which acts in an opsoninization way by attaching to microbial cell surfaces and designating them to be recognised by the complement system and phagocytes. Endocytic pattern-recognition receptors are found at the phagocyte surface and play an important role in distinguishing specific, highly conserved structures on a microbial cell and facilitating the uptake of the pathogen into lysosomes in which they are destroyed. The Toll-like receptor (TLR) family is one of the important examples of signalling receptors which identify specific highly conserved microbial structures and stimulate signal-transduction cascades that lead to the expression of a diversity of immune-response genes, including inflammatory cytokines (Medzhitov and Janeway, 2000a).

In keeping with the participation of PRRs in detecting different microbial structure, another functional classification has deepened our understanding of these molecules. Thus, two functional categories of PRRs can be classified, nonsignalling and signalling PRRs. Lipopolysaccharide-binding protein (LBP) is a good example of the former and TLR of the signalling receptor (Kaisho and Akira, 2006).

In contrast to trying to identify every possible antigen, a relatively small number of innate immune receptors recognize a few highly conserved structures which exist in numerous microorganisms (Janeway, 1989; Akira *et al.*, 2006). These microorganism-specific structures are often called pathogen-associated molecular

patterns (PAMP) and the innate immune receptors which are responsible for recognizing them are PRRs (Janeway and Medzhitov, 1998). There are many well known examples of PAMP in the microbial kingdom such as bacterial lipopolysaccharide, peptidoglycan, lipoteichoic acids, mannans, bacterial DNA, double-stranded RNA (Medzhitov and Janeway, 2000a).

Although different examples of PAMPs vary in their chemical composition, they have three main characteristics in common. Firstly, PAMPs are only considered to be a microbial structure not present with host molecules or cells. Secondly, PAMPs are usually vital for either the pathogenicity or the survival of a particular microbe. Finally, PAMPs of a given microbial class are usually consistent and common within the entire microbial class (Medzhitov and Janeway, 2000a; Beutler, 2004b; Akira *et al.*, 2006; Horner, 2006). Being vital for the survival of the microbe provides a theoretical assurance in keeping the mutation rates at a diminutive level which does not allow PAMPs to escape from immune recognition (Teixeira *et al.*, 2002; Beutler, 2004b). Noticeably, PAMPs are not exclusive molecules for pathogens but are also found on non-pathogens such as normal gut flora (Horner, 2006). Therefore, it seems more accurate to identify them as microorganism-associated molecular patterns (Cohen, 2002; Kaisho and Akira, 2006).

1.2.2 Functional role of endotoxin

Most Gram-negative bacteria have a mixture of amphiphilic molecules within the outer membrane. Of these molecules, endotoxin is considered to be of microbiological and immunological importance as a principal surface antigen (Dixon and Darveau, 2005). From the host part, the presence of endotoxin, as a PAMP, distinctly signifies the presence of Gram-negative bacteria as no other PAMP from other types of organisms can do (Medzhitov and Janeway, 2000b; Medzhitov and Janeway, 2002).

Basically, endotoxin contributes to the outer membrane of the Gram-negative bacteria, structurally as a supportive macromolecular domain of the cell envelope and functionally as is highly anionic and acts as selective permeability barrier to molecules of either negatively charged and/or hydrophobic domains (Horn *et al.*,

1996; Lerouge and Vanderleyden, 2002), adsorption receptor of some bacteriophages and more obviously is its toxicity and immunogenicity to many higher organisms (Leive *et al.*, 1968; Morrison and Leive, 1975).

It has been recognized for a long time that highly purified bacterial endotoxin is very toxic when it is injected systemically and its consequent effect is a pathophysiologic condition similar to the septic shock condition seen in Gram-negative bacteraemia (Rietschel *et al.,* 1994; Horn *et al.,* 1996). Furthermore, the capability of endotoxin to stimulate host responses is not inactivated at very high temperature. For example, it is only considered to be inactive after 4 hours at 160°C (Radon, 2006). In this regard, there is a general agreement that endotoxin functions as a molecule that provides awareness to the immune system of the presence of Gram-negative bacteria within the body (Horn *et al.,* 2000).

Some early doubts about the way endotoxin might interact with host cells in a specific way, had been clarified by the substantial progress that has been achieved in the field of identifying the molecules responsible for sensing of, and interacting with endotoxin. These studies established the idea of endotoxin recognition via specific host receptors (Schletter *et al.*, 1995b; Beutler and Poltorak, 2001). So, it is scientific to postulate that any substance which generates a biological effect at very low concentration often does so by connecting with a precise receptor that works as a signal-amplifier (Beutler and Rietschel, 2003).

Unlike many protein exotoxins which act by killing host cells or by inhibiting cellular functions, endotoxin instead acts non-intrinsically and fundamentally via its Lipid A moiety. As a prototypical cell inducer, it is capable of stimulating a potent inflammatory immune response which is entirely conferred by the host itself (Alving, 1993; Rietschel *et al.*, 1994; Beutler and Poltorak, 2001). Nevertheless, bacterial components other than endotoxin are acknowledged to be endotoxic-like biological inducers such as bacterial exotoxins, lipopeptides, peptidoglycan, lipoteichoic acid and double-stranded (ds)RNA (Beutler and Rietschel, 2003). Most of the time, the usual outcome is successful antimicrobial protection rather than lethal toxicity.

On the other hand, low doses of endotoxin showed a beneficial role in term of stimulate immune response and enhance resistance to infections and malignancies

(Rietschel *et al.,* 1994; Schletter *et al.,* 1995b). However, in a situation like endotoxin hypersensitivity, even low doses may be harmful. Bacterial exotoxins, chronic infection and tumors are considered as a contributing factors for endotoxin hypersensitivity (Rietschel *et al.,* 1994).

Macrophages are of fundamental importance to the immune response against endotoxin (Michalek *et al.,* 1980; Freudenberg *et al.,* 1986). Basically, because they are the main source of tumor necrosis factor alpha (TNF-α) obtained by endotoxin induction *in vivo* (Mannel *et al.,* 1980). However, endotoxin can stimulate other host cells such as endothelial cells, smooth muscle cells and neutrophils, to produce and release mediators of endogenous origin. These include bioactive lipids (e.g., platelet-activating factor and thromboxane A2), reactive oxygen species (e.g., nitric oxide), and, in particular, proteins such as interleukin-1(IL-1), IL-6, and TNF-α. As a rule, the more endotoxins are released, the more of these mediators are released in a consequential pattern of the pathophysiological reactions (Rietschel *et al.*, 1994; Schletter *et al.*, 1995b; Beutler and Poltorak, 2001). TNF-α, is one of the main contributors to the lethal consequence of endotoxin exposure and its production has been considered as a biologically significant endpoint of the endotoxin response. For this reason, macrophages are the most suitable target for the study of endotoxin effect (Beutler *et al.,* 1985; Poltorak *et al.,* 2000).

In both health and disease, endotoxin is in a continuous dialogue with the host which develops several mechanisms to deal with various amounts of endotoxin. These responses depend on physiological, biochemical and biophysical approaches with the aim of diminishing or removing endotoxin effects. Trying to understand each individual strategy in isolation from the others is problematical since they evidently overlap, whether they take place extracellularly or intracellularly (Elsbach, 2000).

As a free or complexed form, endotoxin is released from Gram-negative bacterial cell either at multiplication or after dying (Darveau, 1998). Consequently, interaction events take place between endotoxin and the host immune system via a cascade of molecules either at the site of local inflammation or systemically in the bloodstream. Some of these molecules are located outside the cell helping in recognition of

endotoxin while the other are found inside the host cell leading to the activation of inflammatory immune response (Antal-Szalmás, 2000).

Most of the rest of this section, will concentrate on the extracellular events that occur either outside or on the host membrane which include a receptors complex (discussed below) and finally contribute to the inflammatory immune response.

1.2.3 LBP

Lipopolysaccharide binding protein (LBP) is proposed to be the first host receptor involved in LPS recognition leading to inflammatory immune response (Schumann *et al.,* 1990).

Lipopolysaccharide binding protein is a 50-kDa polypeptide mainly produced by hepatocytes and is released as a glycoprotein into the blood circulation (Schumann *et al.,* 1990). Other LBP producer cells have been recognized including; epithelial cells of the skin, the lung, the intestine and human gingival tissues in addition to the small muscle cells of the lung arteries, heart muscle cells and renal cells (Su *et al.,* 1994; Dentener *et al.,* 2000).

Various human cell line and animal models provide clear evidence that LBP is a secretory class 1 acute-phase protein (Zweigner *et al.,* 2006). It is considered to be a member of the lipid transfer/lipopolysaccharide binding protein (LT/LBP) family which also include bactericidal permeability increasing protein (BPI), the cholesteryl ester transfer protein (CETP) and the phospholipid transfer protein (PLTP). The basic activity of these molecules involves their ability to bind both specific lipid substrates and interact with bacteria and/or lipoproteins (Zweigner *et al.,* 2006).

LBP is principally present in human serum at concentrations of 5-15 µg/ml (Froon *et al.,* 1995; Zweigner *et al.,* 2001). This concentration of LBP is increased by 50-100 fold in inflammation status as a result of either the introduce or presence of microbial stimuli (Schumann *et al.,* 1990; Schletter *et al.,* 1995b; Tobias *et al.,* 1999).

Basically, LBP acts as an amplifying system that alerts host immune cells to the presence of minute quantities of LPS (Elsbach, 2000).

At physiological concentrations, LBP fundamentally enhances the close contact between LPS molecules, in either free or aggregated form, and other recognition molecules either in the serum, such as high density lipoprotein (HDL) and soluble CD14, or on cells, such as membrane CD14 (Yang *et al.*, 1998; Freudenberg *et al.*, 2001).

The potential consequences for such close contact include cell activation via CD14 or neutralization of LPS by means of HDL. Accordingly, the way the host responds to LPS is determined to some extent by the rate of either process. Kinetic studies revealed in particular that LPS/LBP complexes combine with CD14 more rapidly than LPS transfers to HDL . This observation proposes a probable priority situation in which LPS initially triggers immune cells earlier then it is neutralized in order to avoid over-stimulation of the response (Heumann and Roger, 2002).

The presence of LBP in a serum-free cell system increases the endotoxin-mediated stimulation of CD14-positive cells 100- to 1000-fold. It has been shown that LBP transfers LPS to sCD14 and this can lead to the activation of mCD14-deficient cells such as endothelial and epithelial cells (Zweigner *et al.,* 2006).

One study assumed that a single LBP molecule has an ability to transfer hundreds of LPS molecules to CD14 without being consumed by this reaction (Tobias *et al.,* 1995). Another recent study showed that LBP acts as an integral part of stable trimolecular complexes in which it interacts with LPS and CD14. This enables monocytes, for example, to respond to concentrations of LPS as low as 10 pg/ml (Thomas *et al.,* 2002).

In laboratory animals, removal or reduction of LBP levels leads to almost complete elimination of LPS-induced toxicity which shows the need for LBP to transfer LPS to its receptor complex *in vivo*. Many studies have shown that compared with normal wild type mice, LBP-deficient mice were unable to generate a successful early inflammatory response to Gram-negative bacteria such as *S. typhimurium*, *Klebsiella pneumoniae*, and *E. coli* as a result of increased lethality because of bacterial overgrowth (Opal *et al.,* 1999; Branger *et al.,* 2005). In the same context, one study found that anti-LBP antibodies provide protection for mice from low LPS dose to endotoxaemic shock (Gallay *et al.,* 1994). Nevertheless, the highly elevated level of

LBP may display protective effects against LPS by way of reduction of the monocytes responsiveness to LPS (Freudenberg *et al.*, 2001; Zweigner *et al.*, 2001). One study showed that introducing recombinant LBP in a high dose of 100μg per mouse improved the survival rate of mice injected with either fatal amounts of LPS or live *E. coli* O111:B4 (Lamping *et al.*, 1998).

There are potential inhibitory mechanisms of LBP that could be mentioned; one is that LBP has the capability to neutralize bioactive LPS molecules by transferring them to plasma lipoproteins (Wurfel *et al.,* 1994; Vesy *et al.,* 2000). The second probable mechanism is that although sometimes LPS-LBP complexes are internalized by mCD14, they contribute to diminshed LPS signalling since the complexes are aggregated in large forms (Gegner *et al.,* 1995). Another inhibitory mechanism came from a recent study revealing that LBP can dissociate the attachment between LPS and membrane-bound CD14 (mCD14) to inhibit monocyte responses to LPS (Thompson *et al.*, 2003). Therefore it is tempting to speculate that there is a concentration-dependent style for LBP activity in which LBP can mediate signalling of LPS at all LBP concentrations. Whereas, the inhibitory effects need rather high LBP concentrations to be significant (Thompson *et al.,* 2003).

In addition to the binding of LBP to LPS, two studies provide evidence that LBP has an additional ability to bind intact bacterial cells of *Salmonella* spp. and *K. pneumoniae* leading to the clearence of these bacteria via phagocytosis (Wright, 1989; Fan *et al.*, 2002). Although LBP was traditionally regarded as a binding receptor committed to Gram-negative bacteria (Tobias *et al.,* 1986), recent studies indicated that LBP has the capability to attach to other bacterial compounds leading to stimulation of innate immune response. Most of these compounds were found to be amphiphilic in nature, such as glycolipids or lipoproteins, lipoteichoic acid (LTA) of different Gram-positive bacteria and LTA-like glycolipids isolated from spirochaetes. Apparently, since there are different sources of LBP in tissues such as lung and intestine and due to the soluble character of LBP, it has the ability as an important defence molecule to distinguish a diversity of bacterial pathogens prior to the establishment first contact with the immune mechanisms (Schroder and Schumann, 2005). These observations suggested that apart form the classical role of

LBP in binding and transfer LPS to the immune cells, it has a more complex immunomodulatory capability at high concentrations (Zweigner *et al.,* 2006).

In regard to the structural-functional features of LPS, the early evidence that different LPS conformations interrelate in different ways with specific receptor complexes came from investigations related to LBP. These investigations revealed that the *E. coli* conical shape LPS which has a Lipid A structure consisting of six acyl groups in an asymmetrical model, attaches firmly to LBP, whereas a more cylindrically formed LPS, such as that of *P. gingivalis* with five asymmetrically dispersed acyl groups, attaches weakly to LBP (Cunningham *et al.,* 1999). For example, LPS from both *Helicobacter pylori* and *Porphyromonas gingivalis* are bound by LBP with low affinity, less than 10-100 times than *E. coli* (Cunningham *et al.,* 1996).

1.2.4 CD14

CD14, formerly known as a monocyte-specific antigen (Van Amersfoort *et al.,* 2003), is an important receptor since once LBP-LPS complex is accomplished, it is transferred to either soluble or membrane-bound CD14 (sCD14 or mCD14). Therefore, binding to CD14 is most likely to be the second step in the LPS-signalling process (Dixon and Darveau, 2005).

In fact, forming a complex of CD14-LPS radically decreases the necessary concentration of LPS to mediate signalling by 100 to 1000 fold compared to LPS alone (Landmann *et al.,* 2000). However, CD14 does not show identical affinity for all kinds of LPS. For example, one interesting binding study demonstrated that *P. gingivalis*, *H. pylori* and *E. coli* LPSs bind to CD14 in different way, and the amount of *P. gingivalis* LPS needed to gain half-maximum binding to CD14 was about 10-fold more than that of *E. coli* LPS (Cunningham *et al.,* 1999).

CD14-deficient cell lines like 70Z/3 which is unresponsive to LPS, regain its ability of sensing LPS after being transfected with human or rabbit CD14 (Lee *et al.,* 1992). Furthermore, genetically CD14-deficient human monocytes showed low affinity for LPS binding (Couturier, 1991).

CD14 appears to work via concentrating-LPS in a manner which provides a kind of charged surface which promotes LPS interactions in an electro-static manner (Wright, 1995). This carrier role of CD14 is far from being able to distinguish between different microbial ligands (Delude *et al.*, 1995; Wright, 1995; Cunningham *et al.*, 1999). Indeed, it seems to be a step of the collection of sufficient LPS for recognition by a second receptor (Funda *et al.,* 2001). To date, much of the accumulating evidence indicate that CD14 can not activate the LPS-cell signalling since it is not a transmembrane protein therefore it does not have an intracellular tail to deliver the signal (Beutler and Rietschel, 2003; Marshall, 2005).

In addition to LPS, CD14 can bind to a broad spectrum of diverse microbial structures, suggesting the potential central role of CD14 as a PPR (Pugin *et al.,* 1994; Freudenberg *et al.,* 2001). Although it is far from accurate to speculate that this sheds light on the fact that the clinical picture of sepsis is very similar whether it is caused by Gram-negative or Gram-positive bacteria, or even by fungi, it indicates that CD14 is probably a common receptor for many different microbial structures (Landmann *et al.,* 2000). In keeping with this notion, CD14 functions as a lectin-like receptor to potentially identify a variety of sugar or glycolipid patterns in microorganisms (Heumann and Roger, 2002).

CD14 is also considered as a receptor for peptidoglycan of Gram-positive cell wall (Pugin *et al.,* 1994). Not only that, but a recent report indicated the distinctive importance of mCD14 as a sensor for fimbriae from *P. gingivalis* (Hajishengallis *et al.,* 2006). Recently, other functional roles for CD14 apart from endotoxin signalling have been established. These include the recognition and phagocytosis of apoptotic cells, stimulation of B cells and control of T cell activity (Jacque *et al.,* 2006).

In a manner consistent with the structural effects of endotoxin on the signalling process, one interesting study shows that both fully acylated LPS and enzymatically deacylated LPS (dLPS) can interact efficiently with CD14-LBP complexes but only the former can go further to mediate effective LPS signalling while dLPS can not (Kitchens *et al.*, 1992). This observation suggests that the different effects of these types of LPS takes place subsequent to the CD14-LPS interaction where dLPS can not stimulate signalling since it is missing the required structural information

(acyloxyacyl groups) (Kitchens *et al.,* 1992). In the same context, data from two studies indicated that neither human nor murine CD14 have the ability to discriminate between different Lipid A molecules. This supports the suggestion that this kind of species-dependent discrimination of Lipid A structures happened at a step beyond LPS/CD14 interaction (Delude *et al.,* 1995; Muroi *et al.,* 2002). Nevertheless, epitope-mapping studies demonstrated that CD14 selectively binds *E. coli, P. gingivalis* and *H. pylori* LPSs via hydrophilic domains of the amino-terminal region of CD14 and the differences in CD14-affinity might be attributable to the fact that *H. pylori and P. gingivalis* Lipid As are monophosphorylated and have few but longer-chain fatty acids (Cunningham *et al.,* 2000).

Moreover, studies with LPS isolated from *E. coli, S. abortus* ss *equi, S. minnesota, P. aeruginosa, N. meningitidis, Bacteroides fragilis,* and *Rhodobacter sphaeroides* illustrated that the presence of O-antigen, six acyl chains in the Lipid A, and two Kdo units are important requirements for CD14-dependent responses since wild-type LPSs which lack an O-antigen and including a short core and one Kdo or even a phosphorylated Kdo stimulate responses independent of CD14 (Gangloff *et al.,* 1999). In keeping with this, recent study has shown that there are a significant differences between $CD14^{+/+}$ and $CD14^{-/-}$ mouse macrophages in their ability to bind smooth *E.coli* LPS and its various partial structures which do not have an increasing amounts of carbohydrate. In $CD14^{+/+}$ cells, sensitivity to different partial structures of LPS declines in as far as 500-fold below that to smooth LPS. Whereas $CD14^{-/-}$ cell are incapable to differentiate between smooth LPS and its partial structures. Additionally, $CD14^{-/-}$ macrophages are 150,000-fold less sensitive than $CD14^{+/+}$ macrophages to smooth *E.coli* LPS. A comparable capability to discriminate between different LPS structures of other bacteria such as *Bacteroides fragilis* and *Salmonella abortus* are observed for $CD14^{+/+}$, but not $CD14^{-/-}$, macrophages. This would probably make CD14 a highly specific receptor in term of sensitivity and ability to distinguish between various LPS ligands (Gangloff *et al.*, 2005)

1.2.4.1 Membrane CD14 (mCD14)

mCD14 is a 55-kD glycosyl-phosphatidylinositol (GPI) anchored membrane protein (Lee *et al.,* 1992; Lee *et al.,* 1993). Numerous host cell types express mCD14 on their surface. It is found mainly on myeloid cells such as monocytes, macrophages, polymorphonuclear but it is also present on nonmyeloid cells such as B cells, gingival fibroblasts, mammary cells, placental trophoblasts, dendritic cells, respiratory epithelial cells, cornea, ciliary body epithelial cells, uroepithelial, smooth muscle cells and pancreatic islet β cells (Sugawara *et al.*, 1998; Antal-Szalmás, 2000; Funda *et al.*, 2001; Bas *et al.*, 2004; Jersmann, 2005). Furthermore, human intestinal epithelial cells have the ability to either express mCD14 or liberate sCD14 (Funda *et al.,* 2001). The expression of mCD14 on the host cells listed above was shown by many investigators to be through endogenous production rather than transient expression by the virtue of sCD14 adsorption (Jersmann, 2005). In term of mCD14 molecules presence, there are an estimated 30,000 - 45,000 mCD14 molecules per single monocyte based on monoclonal antibodies binding to the same epitope on human monocytes (Van Voorhis *et al.*, 1983; Vasselon *et al.*, 1997). However, another very thorough analysis using both reference beads and scatchard analysis estimation study reported the CD14 number to be about 110,000 molecules per monocyte (Antal-Szalmas *et al.*, 1997).

Apart from its role in LPS-signalling, another function of mCD14 is its role as a signalling receptor for Interleukin 2 (IL-2) in monocytes. This distinctive functional role has been clearly revealed by using the CD14-deficient human U937 promonocytic cell line which is unresponsive to IL-2. These cells gained the ability to respond to IL-2 after being transfected with the gene for human CD14 (Bosco *et al.,* 1997).

Several studies have confirmed that mCD14 also acts as an uptake receptor for LPS internalization (Schiff *et al.,* 1997; Kitchens *et al.,* 1998; Poussin *et al.,* 1998; Latz *et al.,* 2002). In the same context, anti-CD14 murine monoclonal antibodies (anti-CD14 mAb) inhibit LPS–LBP complexes from binding to CD14 in mice experiment and lead also to the low production of different proinflammatory cytokines (Leturcq *et al.,* 1996). However, it is observed that specific anti-CD14 mAb can inhibit LPS-

signalling process without affecting the LPS-internalization whereas other anti-CD14 mAb inhibit LPS-internalization without altering LPS-signalling effects (Gegner *et al.,* 1995). Needless to say this means CD14 has a potential dual role in two different immune mechanisms (as a receptor in signalling process and as a scavenger in internalization process) otherwise the first impression may give a false conclusion that CD14 has two a slightly opposite functions at the same time (Heumann *et al.,* 2003).

1.2.4.2 Soluble CD14 (sCD14)

Two forms of sCD14, a 56-kDa and a 48-kDa molecule, have been identified in normal human circulation based on both the molecular weight and mobility (Antal-Szalmás, 2000). sCD14 serum concentrations vary between 3–6 mg/ml (Bazil *et al.*, 1986; Schletter *et al.*, 1995a). It is thought that sCD14 has a considerable ability to mediate the LPS-induced activation of mCD14-deficient cells such as endothelial cells (Kirschning *et al.,* 1998; Furst-Ladani *et al.,* 1999; Bas *et al.,* 2004).

Seemingly, sCD14 works in parallel with LBP in the same concentration-dependent manner. As with mCD14, lower concentrations of sCD14, which apparently are found in extravascular fluids, mediate a beneficial inflammatory immune response. On the other hand, moderate to higher concentrations of sCD14 in blood circulation are found to prevent LPS-induced systemic inflammation (Thompson *et al.,* 2003). In the latter inhibitory function, sCD14 can challenge mCD14 in a competitive way to bind LPS which can reduce the endotoxin activity in the end (Landmann *et al.,* 2000; Bas *et al.,* 2004).

Furthermore, sCD14 acts in a similar way to LBP by enhancing LPS transfer to HDL to neutralize the LPS. This suggests that both LBP and sCD14 may function as shuttle molecules for LPS, mediating both stimulatory and inhibitory effects by the use of mCD14 and HDL, respectively (Heumann and Roger, 2002).

1.2.5 Toll like receptors

Although the complete and exact molecules that recognize endotoxin have yet to be identified, the discovery of Toll-like receptors (TLRs) stand as the main conduit of the endotoxin transduction mechanism (Beutler and Rietschel, 2003). Not only that but the discovery of TLRs has had an equal impact on immunology as the importance of early discoveries of the receptors that serve adaptive immune response, namely the immunoglobulins and the T-cell receptors (Beutler, 2004a). In light of this, it is needless to say that TLR immunobiology currently creates a centre of huge attention more than any immuology research area at the present time (Pandey and Agrawal, 2006).

TLRs are a family of PRR which consist of type 1 integral transmembrane glycoproteins which consist of leucine rich repeat (LRR) units in the pathogen-binding ectodomains (ECD). Signal transfer occurs via intracellular structural components including an almost identical structure in TLR and IL-1 receptor family members, called Toll/IL-1 receptor homologous (TIR) domain which represent the cytoplasmic end. Both ends are linked by a single transmembrane helix (Akira *et al.*, 2006; Kaisho and Akira, 2006; Pandey and Agrawal, 2006). Moreover, the conserved TIR domain is found in many transmembrane and cytoplasmic proteins in plants, worms, arthropods and even bacteria. Interesting all these molecules are found to have a function in host defence, the feature that makes TIR one of the earliest signalling domains known (Aravind *et al.,* 1999).

Up to date, 13 mammalian TLR have been recognized, of which, 11 are known to be expressed in humans (TLRs 1-11) and 12 are expressed in mice (TLRs 1-9 and 11-13). Human TLRs are so called because they have a common sequence similarity with toll protein in *Drosophila* (Zhang *et al.*, 2004; Kaisho and Akira, 2006). (see Figure 1.2)

Drosophila toll contributes to both dorsal–ventral patterning in fly embryos and fly immunity against fungi. Domain similarity in the cytoplasmic area among *Drosophila* toll and mammalian IL-1 receptor drove the research efforts to discover a human Toll which was later designated to be TLR4 (Medzhitov and Janeway, 2000a; Kaisho and Akira, 2006; Pandey and Agrawal, 2006). Although murine TLR provide

a very useful tool in this field, there are a number of reasons why care should be taken into account when comparison are made between human and murine TLRs in term of functionality and regulatory role. First, there are several variations between humans and mouse strains in their response to specific PAMPs, like LPS for example. Second, the regular use of tumour cell lines as a model of TLR regulation does not show as close a correlation with what happens in primary cells *in vitro* and *in vivo*. Third, even for a specific cell line from a specific species, laboratory experimental conditions may add an additional limit to cross-species comparison (Rehli, 2002).

According to both size and inner structure of the ectodomains, human TLR can be divided into two main groups: TLR1–TLR6 and of TLR7–TLR9 (Chuang and Ulevitch, 2000; Du *et al.*, 2000). Additionally, TLRs can be divided according to their cellular location. For example, TLR1, TLR2 and TLR4 are expressed on the extracellular surface, as verified by positive staining cell with surface antibodies. Whereas, TLR3, TLR7, TLR8 and TLR9 have been demonstrated to have an intracellular expression in cellular components such as endosomes (Takeda and Akira, 2005; Akira *et al.*, 2006).

Identification of many of the TLR ligands was revealed via screening experiments of different kinds of PAMPs in human embryonal kidney (HEK293T) cells which had been transfected with different TLRs. HEK293T cells are a very useful tool in this regard since they do not express any of the TLRs (Janssens and Beyaert, 2003). In light of this it has become clear that TLRs have the ability to recognize a wide range of PAMP ligands varying from protozoa, to bacteria, to fungi and to viruses (Beutler, 2004a). Those ligands can be divided into three categories based on their structure: lipid, protein, and nucleic acids (Kaisho and Akira, 2006). For example, TLR1, 2 and 6 recognise PAMPs of lipid or protein origin and TLR7, 8 and 9 recognise nucleic acids (Akira *et al.,* 2006).

In addition to microbial molecules and non-self ligands, TLRs have the capability to recognize several endogenous ligands as well. Among these are hyaluronic acid, fibrinogen, fibronectin, heat-shock proteins, beta defensins and heparin sulphate (Akira *et al.*, 2006; Kaisho and Akira, 2006; Pandey and Agrawal, 2006)

However, according to the current picture from different studies regarding the relation between TLR and their ligands, it is plausible to conclude that there is no exclusive single TLR dedicated to one group of pathogens. For example, PAMPs of Gram-negative origin can be recognized by both TLR2 and TLR4. In addition, one TLR can be signalled by different unrelated PAMPs from different groups of pathogens. For example, both viral components and Gram-negative LPS are recognised by TLR4 (Janssens and Beyaert, 2003).

Two main regulatory functional roles of TLRs are currently recognized, the stimulation of innate inflammatory response and the establishment of adaptive immunity (Kaisho and Akira, 2006; Pandey and Agrawal, 2006). TLR2 and TLR4 have received the most attention due to their relationship with bacterial endotoxin (Schaub *et al.,* 2006). These two receptors are discussed below in details.

1.2.5.1 TLR2

Although the picture of the functional role of TLRs is far from being completed, TLR2 has an extraordinary position among other members of the human TLR family since it has the capacity to identify a wide range of PAMPs from different kind of microorganisms including those from Gram-positive bacteria, Gram-negative bacteria, mycobacteria, fungi, viruses and parasites (Raetz and Whitfield, 2002; Texereau *et al.*, 2005). Examples of the range of PAMPs recognized by TLR2 include lipoproteins/lipopeptides, peptidoglycan, lipoteichoic acid, lipoarabinomannan from mycobacteria, glycosylphosphatidylinositol from *Trypanosoma cruzi*, a phenol-soluble modulin from *Staphylococcus epidermis*, zymosan from fungi and glycolipids from *Treponema maltophilum* (Takeda and Akira, 2005).

Furthermore, the way that TLR2 is distributed through the human body indicates its central role as a very first line of antimicrobial defence. The cells of hematopoietic origin especially peripheral blood leukocytes such as monocytes, macrophages, granulocytes and dendritic cells exhibit the highest expression level followed by cell populations from the spleen and lung and other kind of cells that have a role in acute host defence (Yang *et al.,* 1998; Texereau *et al.,* 2005).

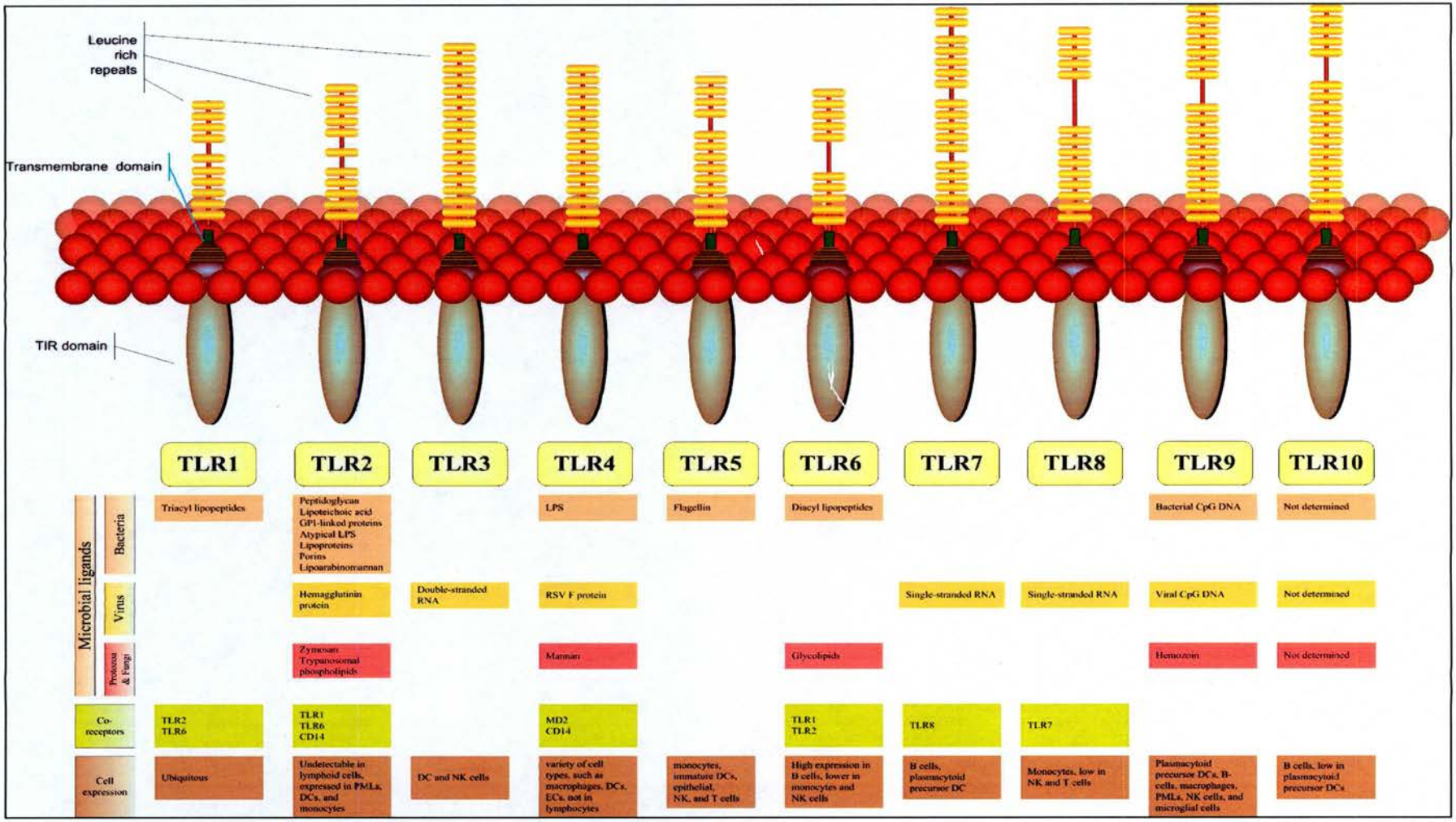

Figure 1.2 Human toll like receptors

There are several proposals for the way TLR2, as a single molecule, has the distinctive capacity to identify a broad range of PAMPs. One explanation proposes that TLR2 is considered to form a multi-signalling TLR receptor complex by heterodimerization with other TLRs such as TLR1, TLR6 or other TLRs (Ozinsky *et al.*, 2000; Takeda and Akira, 2005; Texereau *et al.*, 2005; Triantafilou *et al.*, 2006). One piece of evidence supporting this mechanism is the observation that TLR6-deficient mice do not exhibit any inflammatory activity against diacyl lipopeptides of *Mycoplasma* (Takeda and Akira, 2005). Although, the same kind of cells demonstrated normal inflammatory response towards triacyl lipopeptides of Gram-negative bacteria (Takeuchi *et al.,* 2001). The vice versa situation is applicable, in which TLR1-deficient mice demonstrated a normal response to the diacyl lipopeptides and weakened response to the triacyl lipopeptides (Takeuchi *et al.,* 2002). This shows the potential functional association between TLR1 and TLR6 on one hand and TLR2 on the other in a way that allow the formation of such heterodimer complex to differentiate between diacyl or triacyl lipopeptides (Takeda and Akira, 2005). Moreover, TLR1 shows its capacity to be involved in the signalling process against outer surface lipoprotein of *Borrelia burgdorferi* (Alexopoulou *et al.,* 2002). The other potential mechanism of TLR2 activity is demonstrated by the model in which TLR2 act in collaboration with unrelated receptors other than TLR to recognize different PAMP structures (Gantner *et al.,* 2003; Triantafilou *et al.,* 2006). The observation that a receptor such as CD36 can assist TLR2 activity in response to diacylated lipoproteins supports this notion (Triantafilou *et al.,* 2006). In addition, other observation have shown TLR2 in collaboration with one of a lectin family receptor, dectin-1, in the recognition of β-glucan, a fungal cell wall component (Takeda and Akira, 2005).

To simplify the continuous debate of whether TLR2 or TLR4 is the physiologically significant LPS receptor, it is important to evaluate every experimental approach in this regard in terms of cell type used and LPS source (Tapping *et al.,* 2000). Primarily, TLR2 was acknowledged as an LPS receptor by the result of overexpression experiments where TLR2 was stimulated with commercial preparations of LPS (Kirschning *et al.,* 1998; Yang *et al.,* 1998). These findings were confirmed by several studies (Aliprantis *et al.*, 1999; Brightbill *et al.*, 1999;

Hirschfeld *et al.*, 1999; Means *et al.*, 1999a; Schwandner *et al.*, 1999; Flo *et al.*, 2000). In view of such an approach, THP1-CD14 cells that have a significantly high expression level of TLR2 compared with TLR4, were found to demonstrate high sensitivity to impure commercial preparations of LPS (Tapping *et al.*, 2000). Nevertheless, two important studies have demonstrated that this kind of TLR2 activity is attributable to impurities in the LPS that were revealed to be protein or lipoprotein contaminants. LPS re-extraction methods eliminate the LPS signalling through TLR2 and confer a TLR4 response (Brightbill *et al.*, 1999; Hirschfeld *et al.*, 1999; Hirschfeld *et al.*, 2000). At the same time these findings shed more light on the capacity of TLR2 to recognize LPS-contaminated endotoxin protein (Takeuchi and Akira, 2002). Although the contribution of TLR2 as a signal transducer for such LPS-contaminants protein or microbial lipoproteins in general does not exclude the contribution of other TLR family members in term of combined-TLR signalling (Brightbill *et al.*, 1999). In the same context, Chinese hamster ovary (CHO) cells which are known for their sensitivity to LPS (Golenbock *et al.*, 1993; Delude *et al.*, 1995; Heine *et al.*, 1999), have a nonfunctional version of TLR2 and they showed a normal response to LPS isolated from *Salmonella minnesota* R595 (Heine *et al.*, 1999; Takeuchi *et al.*, 2002).

On the other hand, it is well documented that TLR2 has the capability to recognize LPS structures from bacterial species other than Enterobacteriaceae such as *Leptospira interrogans*, *Porphyromonas gingivalis, Helicobacter pyroli* (Hirschfeld *et al.*, 2001; Werts *et al.*, 2001; Smith *et al.*, 2003), *Rhizobium species* Sin-1, *Legionella pneumophila* (Girard *et al.*, 2003), *Bacteroides fragilis* NCTC-9343, *Chlamydia trachomatis* LGV-1 and *Pseudomonas aeruginosa* PAC-611 (Erridge *et al.*, 2004b). It was also observed that LPS from *Bacteroides fragilis* and *Pseudomonas aeruginosa* were able to produce a signal in TLR4-deficient macrophages isolated from C3H/HeJ mice (Delahooke *et al.*, 1995; Girard *et al.*, 2003). In general, these organisms have different structural features from that of classical LPS, such as that of *E.coli* (see section 1.1.4), which is shown to be recognized by TLR4 instead. These variations, which include the number and the distribution of acyl chains in the Lipid A component, seemingly lead to a different recognition style (Netea *et al.*, 2002). The multiplicity of a functional role of the

conformation types of LPS have shown that conical and cylindrical types of LPS might attach to and signal different receptor complexes. This was revealed by the findings that *P. gingivalis* LPS of cylindrical shape signals cells via TLR2 but not TLR4, while *E. coli* LPS of conical shape use TLR4 as a signal transducer (Hirschfeld *et al.,* 2001; Netea *et al.,* 2002). Intriguingly, LPS obtained from *P. gingivalis* is abnormal and highly heterogeneous, containing both tetra- and penta-acylated Lipid A structures (Darveau *et al.*, 2004a; Reife *et al.*, 2006). Some reports introduced this LPS as an agonist for TLR2 (Bainbridge and Darveau, 2001; Hirschfeld *et al.*, 2001; Martin *et al.*, 2001) and other reports provided evidences of its ability to be either antagonist (Darveau *et al.*, 2002; Hajishengallis *et al.*, 2002b; Yoshimura *et al.*, 2002; Coats *et al.*, 2003) or even agonist for TLR4 (Tabeta *et al.,* 2000; Ogawa *et al.,* 2002)

Of much interest, LPS of *N. meningitidis* appears to signal via both receptors TLR4 and TLR2. Although, *N. meningitidis* LPS resembles the classical LPS of *E.coli* in which it has six acyl chains, these acyl structures are dispersed equally between the two hexose groups (i.e 3+3 not 4+2). This situation gives it a kind of intermediate conformation between the conical and cylindrical forms, which, hypothetically, could provide the opportunity to attach to either TLR2 or TLR4. This is evident by the findings that high concentration of ultra pure meningococcal LPS still has the capacity to stimulate macrophages of TLR4-deficient C3H/HeJ mice to produce TNF-α but at a level lower than that of control C3H/HeN macrophages. This signifies that both TLR4-dependent and -independent signalling pathways are used (Netea *et al.,* 2002).

Most recent studies have revealed another dimension to the function of TLR2 as one of PPR of the innate immunity. Sutmuller and others have demonstrated that TLR2 has an additional function in controlling the activity of regulatory T cells which are essential cells belong to the adaptive immune system (Sutmuller *et al.,* 2006).

1.2.5.2 TLR4

Many investigators speculated before the discovery of TLRs that there was a receptor which was central to LPS signalling, different from CD14 and expressed on both

CD14-positive or -negative cells (Kitchens *et al.*, 1992; Pugin *et al.*, 1995; Schletter *et al.*, 1995a; Vita *et al.*, 1997; Heumann and Roger, 2002). The current main pieces of evidence supporting the role of TLR4 in signalling the presence of bacterial LPS are as follows. First, the loss of the LPS response due to specific mutant in *lps* locus in mouse. Second, the finding that the *lps* locus is identical to *tlr4* and they code for TLR4. Third, re-extraction of LPS to remove protein contaminants eliminated the ability of LPS to stimulate cells from the LPS non-responder strain of mice, C3H/HeJ. Fourth, none of the human or murine TLR2 transfected cell showed response to either repurifed LPS or protein-free synthetic Lipid A. Finally, the unresponsiveness of TLR4-deficient mice to LPS challenge (Poltorak *et al.*, 1998a; Poltorak *et al.*, 1998b; Hoshino *et al.*, 1999; Hirschfeld *et al.*, 2000; Heumann and Roger, 2002). In general, evidence is accumulating to suggest that, different species of animals may use different Toll like receptor as a signal transducer for LPS (Heine *et al.,* 1999; Takeuchi *et al.,* 1999). In the same context, a TLR4 mutation Asp299Gly that affects the extracellular domain of the receptor and renders it unresponsive to LPS has been transfected into THP-1 human cell line. However, human primary airway epithelial cells or alveolar macrophages from individuals with the Asp299Gly mutation recovered their responsiveness towards commercial LPS when these cells were transfected with the wild-type allele of TLR4 (Arbour *et al.,* 2000). Nevertheless, monocytes from another group of individuals with the Asp299Gly mutations showed LPS response almost identical to that from monocytes from normal individual when they are both challenged with a panel of seven different purified LPS preparations (Erridge *et al.,* 2003). Two possible explanation to resolve this apparent contradiction between these two studies are: first, it may be due to the difference in TLR4 expression between monocytes and airway tissues. Second, it may be due the purity of LPS preparations since the former study used a commercial LPS which is assumed to have a lot of protein contaminants while the later study used a re-purified LPS preparations (Erridge *et al.*, 2003).

In parallel with this, another study found that the TLR4 knockout mice were hyporesponsive to a limited, but not all, set of LPS preparations (Takeuchi *et al.,* 2000). The extracellular domains of both human TLR4 and mouse TLR4 show only 53% similarity, while their cytoplasmic domains are identical by 83% (Anderson,

2000). Moreover, the LD_{50} (defined as the quantity of a substance that causes death of 50% of the animals) for virulent *E. coli* in C3H/HeJ mice, which have a non-functional TLR4, is not more than ten organisms, whereas the LD_{50} for wild-type mice is 10 000 organisms. Accordingly, TLR4 seems to contribute to the protection against septic shock, as a severe complication of an unwanted immune response, more than acting as a cause of it (Brunn and Platt, 2006). Although TLR4 mutations are rare in humans they have been shown to be over-expressed in patients with meningococcal meningitis or septicaemia (Smirnova *et al.,* 2001; Smirnova *et al.,* 2003). In a similar study, the most severe symptoms were seen in 91 patients with septic shock who were genotyped to reveal that they had TLR4 mutations, Asp299Gly and/or Thr399Ile (Lorenz *et al.,* 2002a).

Two kinds of LPS-hyporesponsive mice are known to be *tlr4*-gene deficient. C3H/HeJ mice have a point mutation within the *tlr4* gene and C57BL/10ScCr mice which displayed total absence of the *tlr4*-gene (Poltorak *et al.,* 1998a; Qureshi *et al.,* 1999). These two TLR4 deficient mice are well known as LPS-hyporesponsive (Hoshino *et al.,* 1999). Moreover, mice which have a critical mutational defect in the *Tlr4* locus are noticeably vulnerable to Gram-negative infections. This indicates how the recognition of LPS is so beneficial for the host (O'Brien *et al.,* 1980; Rosenstreich *et al.,* 1982). Functionally, TLR4 acts as the actual signalling piece of the LPS recognition complex to recruit adapter proteins via intracellular Toll/IL-1 receptor domain which lead to the expression of inflammatory gene transcription (Marshall, 2005).

Moreover, recent studies suggested that TLRs tend to be expressed during the immune response event sequentially and in a collaborated order during the course of an infection such as those caused by *Salmonella* and respiratory syncytial virus (RSV). These two pathogens are initially recognized by TLR4 to activate inflammatory responses. After a specific period, the TLR4 activation is down-regulated. At the same time both TLR2 and TLR3 are up-regulated due to the presence of bacterial lipopeptide, and viral dsRNA, respectively (Haeberle *et al.,* 2002; Weiss *et al.,* 2004). Furthermore, recent studies have raised the possibility that the actual number of TLR4 molecules that are engaged is essential in determining the type of signal which is going to be triggered (Visintin *et al.,* 2003; Triantafilou *et al.,*

2004). This seems that a minimum number of TLR4 molecules are required to attach to the receptor complex in order to introduce the maximum cellular activation. If this minimum number of TLR4 is not reached, as in the situation of the LPS antagonists, a decreased activation is observed (Triantafilou and Triantafilou, 2005).

One of the mechanisms suggested to explain the relation between different components of the LPS recognition system, postulates the presence of a so-called proteolytic system between CD14 and TLR4. In this system a polypeptide signal is created as a response to the connection between LPS and CD14, and consequently mediate a response via TLR4 (Wright, 1999). However, this model has high level of uncertainty since CD14 is not known to have proteolytic activity (Beutler, 2000).

As mentioned earlier, the biophysical properties of LPS molecules are proposed to play a role in TLR signalling. Many investigators have suggested that the typical hexa-acyl LPS structure from enterobacteriaceae which have a conical conformation, is the most favorable structure for TLR4 activation, whereas the penta-acyl LPS of a cylindrical conformation have a tendency to signal through TLR2 (Schromm *et al.,* 2000; Netea *et al.,* 2002). It is also found that a tetra-acyl domain of Lipid A alone does not signal human TLR4, while Lipid A does. The situation is quite different with mouse TLR4 which is signalled by both intact Lipid A and tetra-acyl Lipid A (Poltorak *et al.,* 2000). It has been postulated that human TLR4, but not mouse TLR4, is able to ''read'' the LPS structure, evaluate the nature of acyl chains and finally distinguish between Lipid A and tetra-acyl Lipid A (Poltorak *et al.,* 2000). Moreover, uncommon LPS molecules like Lipid A analogues lipid IVa and *Rhodobacter sphaeroides* Lipid A contribute to this TLR4 species-specific variation in which they both abolish the LPS effects in human cells but show LPS- like activity in Chinese hamster ovary fibroblasts expressing CD14 (Lien *et al.,* 2000).

In regard to species-dependent discrimination, two studies found that TLR4 has its own capacity to differentiate between tetra-acylated Lipid A precursor (also called compound 406 or lipid IVa) and *R. sphaeroides* Lipid A. The other explanation for this phenomenon is the ability of TLR4 to distinguish between the conformation of CD14 molecules when they bind different Lipid A molecules (Lien *et al.,* 2000; Poltorak *et al.,* 2000).

Noticeably, many of the studies investigating TLR4 as a LPS-signal transducer picked only classical kind of LPS (sample or control) which are known to signal through TLR4 such as those of *E.coli* and *Salmonella* (see Table 1.1). In fact, more than 90% of all publications on endotoxin were done with endotoxins from enterobacteriaceae (Dehus *et al.*, 2006). Whereas, this kind of investigation require at least more than one LPS control to support the results from both classical and non-classical LPSs. For example, one LPS as a TLR4 positive control and another as a TLR2 positive control since the differences between various kind of LPSs from different species are now considered to be the rule not the exception.

However, TLR4 is not a dedicated receptor for LPS. A recent study has shown that a specific viral protein of Hepatitis C virus is recognised by TLR4 resulting in production of β-Interferon and Interleukin-6 (Machida *et al.,* 2006). Other PAMPs of different structure that have been found to be recognized by TLR4 include the plant diterpene paclitaxel and the fusion protein of respiratory syncytial virus (RSV) (Kurt-Jones *et al.,* 2000; Akira *et al.,* 2006). In addition, TLR4 has been shown to be a signal transducer for endogenous ligands such as heat shock proteins (HSP60 and HSP70), the extra domain A of fibronectins and oligosaccharides of hyaluronic acid, heparan sulfate and fibrinogen (Takeda and Akira, 2005). However, all of these endogenous ligands need exceedingly high concentrations of TLR4 to be activated (Takeda and Akira, 2005). These findings need to be appraised carefully since it is well known that LPS is a very potent immuno-inducer, and therefore, TLR4 can be activated by a very minute amount of contaminating LPS, that might be present in endogenous ligand preparations (Takeda and Akira, 2005).

Most recently, two interesting studies have demonstrated further clarification about the functional role of TLR4. One of them showed for the first time that TLR4 plays a role in the induction of fever in mice (Steiner *et al.,* 2006). The other study also demonstrated for the first time that TLR4 is expressed on both human and murine platelets (Aslam *et al.,* 2006).

Table1.1 Examples of LPS preparations used in different studies

Study		Type of lipopolysaccharide	No of citations
1	(Kirschning *et al.*, 1998)	*Escherichia coli* 0111:B4	488
2	(Poltorak *et al.*, 1998a)	Unknown LPS	2348
3	(Chow *et al.*, 1999)	Unknown LPS	670
4	(Heine *et al.*, 1999)	*Salmonella minnesota* R595	178
5	(Hoshino *et al.*, 1999)	*E. coli* O55:B5 & *S. minnesota* R595	875
6	(Takeuchi *et al.*, 1999)	*E. coli* O55:B5 & *S. minnesota* R595	1137
7	(Yang *et al.*, 1999)	*E. coli*	165
8	(Faure *et al.*, 2000)	*E. coli* K235	210
9	(Hirschfeld *et al.*, 2000)	*E. coli* O111:B4, J5 , and K12	405
10	(Lien *et al.*, 2000)	*S. minnesota* R595	340
11	(Muzio *et al.*, 2000)	*E. coli* 005:B5	304
12	(Poltorak *et al.*, 2000)	*E. coli* 0127:B8	238
13	(Tapping *et al.*, 2000)	*E. coli* & *S. minnesota*	135
14	(Naik *et al.*, 2001a)	*E. coli* 0111:B4	61
15	(Gioannini *et al.*, 2004)	*Neisseria meningitides* & *E. coli*	15
16	(Dehus *et al.*, 2006)	*E. coli* & *Salmonella* sp.	1
17	(Edelman *et al.*, 2006)	*E. coli*	0
18	(Hirschfeld *et al.*, 2001)	*E. coli* & *Porphyromonas gingivalis*	226
19	(Pulendran *et al.*, 2001)	*E. coli* & *P. gingivalis*	156
20	(Hajjar *et al.*, 2002)	*E. coli* & *Pseudomonas aeruginosa*	122
21	(Lorenz *et al.*, 2002b)	*E. coli* & *Bacteroides fragilis*	8
22	(Toshchakov *et al.*, 2002)	*E. coli* & *P. gingivalis*	256
23	(Erridge *et al.*, 2004b)	*E .coli* , *Yersinia pestis*, *P. gingivalis*, *B. fragilis*, *Chlamydia trachomatis*, *Ps. aeruginosa*	27
24	(Mancuso *et al.*, 2005)	*B. fragilis* & *Salmonella enterica*	4

1.2.6 Myeloid differentiation protein 2 (MD2)

Myeloid differentiation protein 2 (MD-2) is a homologue of MD-1, a specific secretory protein found in B-cells (Janssens and Beyaert, 2003). It is a small cysteine-rich glycoprotein which is known to join with the ectodomain of TLR4. TLR4 does not have the capacity to transduce LPS signalling unless it is attached to MD-2 (Shimazu *et al.*, 1999a). It has also been revealed that if MD-2 gene expression is disrupted in mice, it leads to abolish the LPS signalling thus proving the importance of MD-2 for the function of TLR4 (Schromm *et al.*, 2001; Nagai *et al.*, 2002b; Nagai *et al.*, 2002a). Moreover, MD-2 $^{-/-}$ mice were found to be hyporesponsive to LPS (Nagai *et al.*, 2002a)

To this end, it is widely believed that cellular activation by LPS necessitates the consequential linkage of a trimeric receptor complex containing mCD14, TLR4, and the accessory adapter protein MD-2. This protein complex stimulates an intracellular signalling pathway via kinase cascades and the transcription factor NF-κB to induce the transcription of several hundred genes that express the phenotype of the consequential systemic inflammatory response (Akashi *et al.*, 2000; Kennedy *et al.*, 2004; Marshall, 2005). The idea of close proximity between LPS and the three proteins complex CD14/TLR4/MD2 is supported by the finding that attachment to CD14 exclusively equips LPS to be linked to the TLR4-MD2 complex (da Silva Correia *et al.,* 2001). It was also found that neither the transfection with TLR4 alone nor MD-2 alone can contribute to the LPS signalling. MD-2, which is an extracellular protein without transmembrane domain, stays attached to the surface of the cell and to the extracellular domain of TLR4 at the same time (Aderem and Ulevitch, 2000; Akira *et al.*, 2001; Erridge *et al.*, 2002). Furthermore, the engagement of LPS/TLR4/MD-2 was found to be in a direct interaction (Akashi *et al.,* 2003). This was revealed by an approach in which the presence of detergent was found to interrupt LPS interaction with CD14 but not with TLR4-MD-2. Moreover, a lipid A antagonist, E5531, was also found to block LPS interaction with TLR4-MD-2 at a concentration 100 times lower than that necessary for blocking LPS interaction with CD14 (Akashi *et al.*, 2003).

Recent study used LPS forms of wild-type and R-mutants of *Salmonella* and *E. coli,* to show that S-form and R-form of LPS stimulate mouse cells in different ways. The R-form activates cell signalling instantly via TLR4/MD2 complex, whereas the S-form activates the same cells via TLR4/MD2 complex with the involvement of LBP and CD14 (Huber *et al.,* 2006). A recent interesting study showed that human, but not murine, TLR4/MD2 complex is able to distinguish between the two types of Lipid A which can be produced by bacterial species like *Pseudomonas aeruginosa* which has the ability to vary between hexa- and penta-acylated Lipid A. Consequently, hexa but not penta-acylated Lipid A, stimulate inflammatory response through human TLR4-MD2 (Hajjar *et al.,* 2002). Furthermore, it was revealed by many investigators that MD-2 has the capacity to distinguish between TLR4 agonists and antagonists (Gioannini *et al.,* 2004). A recent report provides evidence that both a tetra-acylated *P. gingivalis* LPS preparation as well as mutant penta-acylated LPS of *E. coli* antagonise a hexa-acylated *E. coli* LPS at the TLR4 signalling complex of human endothelial cells in a competing manner (Coats *et al.,* 2003) in which MD-2 plays the principal role (Coats *et al.,* 2005).

MD-2 also has a species dependent discrimination activity similar to that of TLR4. One recent study demonstrated that MD-2 can discriminate between *E. coli* and *Salmonella* Lipid A molecules (Muroi *et al.*, 2002). It was also found that only the expression of the following murine complexes either CD14/TLR4/MD2 or TLR4/MD2 allowed THP-1 cells to response to *Salmonella* Lipid A, but neither singular expression of the previous molecules, CD14/TLR4, nor CD14/MD-2 confer the responsiveness to the same Lipid A (Muroi *et al.,* 2002). Moreover, lipid IVa is a useful tool to study the differences between mouse and human in LPS signalling since mouse TLR4/MD-2, but not human TLR4/MD-2, transduce lipid IVa signalling. In light of this, mouse TLR4/human MD-2 (mTLR4/hMD-2) responded to Lipid A from *Salmonella minnesota* but not lipid IVa. Findings of this nature attribute species-specific discrimination activity for hMD-2 as well and demonstrates that hMD-2 has the capacity to regulate the specificity of TLR4. Not only that, but lipid IVa operates as a Lipid A antagonist on mTLR4/hMD-2. This situation needs a physical contact between mTLR4/hMD-2, Lipid A and Lipid IVa (Akashi *et al.,* 2001).

In support of the previous finding, another molecule, Taxol which is an antitumor agent purified from the bark of the Western yew, *Taxus brevifolia,* can signal mTLR4/MD-2, but not hTLR4/MD-2. However, it is found that the HEK293 human cell line transfected with mTLR4/hMD-2 does respond to LPS but not Taxol, although mTLR4/mMD-2 respond to both LPS and Taxol (Kawasaki *et al.,* 2001).

Additionally, an active form of soluble MD-2 (sMD-2) was observed to be secreted by primary cells like immature dendritic cells and also by MD-2-transfected cell lines. sMD-2 seems to have a capacity to bind TLR4 on MD-2 deficient cells and convert these cells to be responsiveness to LPS (Kennedy *et al.,* 2004). Furthermore, a recent study demonstrated that the activity of sMD-2 is diminished within 24-h at physiological temperature unless it is stabilized by LPS treatment in CD14 dependent process. sMD-2/LPS complex was then able to stimulate TLR4 on cells like epithelial cells, which express TLR4 but not MD-2 (Kennedy *et al.,* 2004). It was also found that sMD-2/LPS binding is vastly augmented by molecules such as LBP (Viriyakosol *et al.,* 2001; Kennedy *et al.,* 2004).

Many investigators currently propose that MD-2 has a function similar to that of Spaetzle, the endogenous ligand in *Drosophila* that attaches to Toll and stimulates antimicrobial peptide production. In parallel with this, MD-2 is assumed to go through conformational alteration after binding LPS. This alteration permits MD-2 to activate TLR4 (Gioannini *et al.,* 2004; Visintin *et al.,* 2005).

Another study found that MD-2 can also act with TLR2 to enhance its responsiveness to a wide range of LPS molecules, lipoteichoic acid, Gram-negative bacteria, Gram-positive bacteria and in particular peptidoglycan which does not signal through TLR4/MD-2. The attachment between TLR2 and MD-2 is weaker than that between TLR4 and MD-2 (Dziarski *et al.,* 2001).

Most recently, another novel regulatory protein (PRAT4A or PRAT4B) has been elucidated to be linked with immature form of TLR4, but not MD-2 nor TLR-2. It is thought to regulate the TLR-4 cell surface expression and it is found that PRAT4A knockout eliminated the LPS signalling in cells expressing TLR4/MD-2 (Konno *et al.,* 2006; Wakabayashi *et al.,* 2006).

1.2.7 Intracellular receptors for LPS recognition

A number of the processes in the TLR signalling cascade are similar to that of the IL-1 Receptor (IL-1R) since they share the conserved Toll-like/IL-1 receptor (TIR) domain in their intracellular regions (O'Neill *et al.*, 2003; Akira and Takeda, 2004; Kaisho and Akira, 2006; Pandey and Agrawal, 2006). Obviously, the other part of LPS signalling story is started in that TIR domain as a point of contact which is shared by both the receptor and adaptor molecules and it is located intracellularly inside the cytoplasm (Dunne and O'Neill, 2005; Lasker and Nair, 2006). TIR domains are crucial for TLR signalling processes since a single point mutation in the TIR domain of murine TLR4 eliminates LPS signalling completely (Poltorak *et al.*, 1998a).

To date, there are five adaptor proteins known to be recruited to the TIR domain of TLR: the myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like or TIR-associated protein (MAL/TIRAP), TIR related activator of interferon-β (TRIF), Trif related adaptor molecule (TRAM) and SAM and ARM protein (SARM) (Beutler, 2004a; McGettrick and O'Neill, 2004; O'Neill, 2006). All of these adaptor proteins act as a transducers of signals from the TIR domains downstream to reach the transcription factors that cause inflammatory responses (Beutler, 2004a).

It has been demonstrated that MyD88-deficient mice are hyporesponsive to endotoxin (Lasker and Nair, 2006). Moreover, one study showed that macrophages and splenocytes from MyD88-deficient mice are entirely hyporesponsive to a wide range of Gram-negative, Gram-positive and *Mycobacterium* cell wall components. These results demonstrated that MyD88 is indispensable for the cellular response to bacterial cell wall components (Takeuchi *et al.*, 2000). When MyD88 is being stimulated, it utilizes its amino-terminal death domain to attach the death domain-containing serine-threonine kinases of the IL-1 receptor associated protein kinase 4 (IRAK4) (Akira *et al.*, 2001; Heumann and Roger, 2002; Lasker and Nair, 2006). After activation of IRAK-4, it phosphorylates IRAK-1 which consequently detached from the receptor complex and combine with tumor necrosis factor (TNF) receptor-activated factor 6 (TRAF6). This process eventually leads to activation of nuclear factor (NF-kB) which controls the expression of several genes involved in organizing

the inflammatory response (Athman and Philpott, 2004; Lasker and Nair, 2006; O'Neill, 2006).

The nuclear factor-κB (NF-κB) family is a key participant in regulating both innate and adaptive immunity. They control the expression of cytokines, growth factors, and effector enzymes in response to many receptors involved in immunity such as T-cell receptors (TCRs), B-cell receptors (BCRs) and Toll/IL-1R family molecules (Ghosh and Karin, 2002; Hayden and Ghosh, 2004). In the cytoplasm, NF-κB proteins are found in connection with dedicated inhibitory proteins of NF-κB (IκBs). After stimulation, the IκB proteins are degraded to allow NF-κB proteins to translocate to the nucleus and bind their related DNA binding sites to stimulate the transcription of a large number of genes, including antimicrobial peptides, cytokines, chemokines, stress-response proteins and anti-apoptotic proteins (Li and Verma, 2002).

The cytokines released include pro-inflammatory cytokines (TNF-α, IL-1, IL-6) and chemokines (IL-8) which provoke fever and up-regulate the acute phase response with the production of complement, C-reactive protein and other molecules (Basset *et al.,* 2003). Of these cytokines, TNF-α is considered to be the most fundamental one since the inhibition of TNF activity leads to significant reduction of LPS toxicity (Beutler *et al.,* 1985). On the other hand, it was found that direct administration of TNF-α provokes similar effects to LPS toxicity in normal animals (Tracey *et al.,* 1986). That is why some investigators believe that TNF-α acts as an endogenous mediator of endotoxicity (Beutler and Rietschel, 2003). Subsequently, these cytokines stimulate neighbouring cells to produce chemokines and adhesion molecules in order to attract a range of inflammatory cells into the infection focus. Subsequent responsive cells like macrophages or neutrophils are then stimulated and engulf pathogens via internalizing PRRs. As a result, pathogens are killed by the production of molecules like nitric oxide, reactive oxygen species or defensins. Hence, the inflammatory immune response represents a critical local response to resolve infection. On the other hand, inflammation is a double edged sword since when cytokines are produced in excessive quantity, they cause fatal effect and that is

exactly what happens in endotoxic shock (Weber *et al.*, 2003; Kaisho and Akira, 2006).

All TLR, except TLR3, utilize the MyD88-dependent pathway (Hoebe *et al.*, 2003a; O'Neill *et al.*, 2003; Akira and Takeda, 2004; Kaisho and Akira, 2006; Pandey and Agrawal, 2006). Moreover, genetic investigations utilizing MyD88-deficient mice proved that this factor is vital for the NF-κB-dependent stimulation of the genes responsible for the production of the cytokines TNF-α and IL-6 in response to TLR agonists (Imler and Zheng, 2004).

Furthermore, IRAK4 is central for the responses to IL-1 and ligands that signal different TLRs. IRAK4-knockout animals are entirely insensitive to lethal doses of LPS and are severely weakened in their abilities to resist microbial challenges. Moreover, both deficiency and mutations associated with IRAK4 have been reported in patients suffering from repeated pyogenic bacterial infections (Athman and Philpott, 2004).

Other adaptor proteins, like MAL, revealed its importance since Mal-deficient mice are hyporesponsive to TLR2 and TLR4 stimulation but have normal responses to other TLRs. Additionally, TRIF adaptor protein is considered to be responsible for the induction of IFN-β by the virtue of TLR3 and TLR4 signalling but not TLR2 (O'Neill, 2006).

Figure 1.3 summarises the common steps in inflammatory immune response.

1.2.8 Other immune response against LPS

Either in health or disease, endotoxin is in continuous dialogue with the host which has developed several machanisms to deal with various amounts of LPS depending on different approaches with the aim of diminishing or removing LPS effects. Trying to understand each individual strategy in isolation from the others is problematical since they evidently overlap, whether they take place extracellularly or intracellularly (Elsbach, 2000) The signalling approach was explained in detail in the previous section. Other approaches will be summarized here.

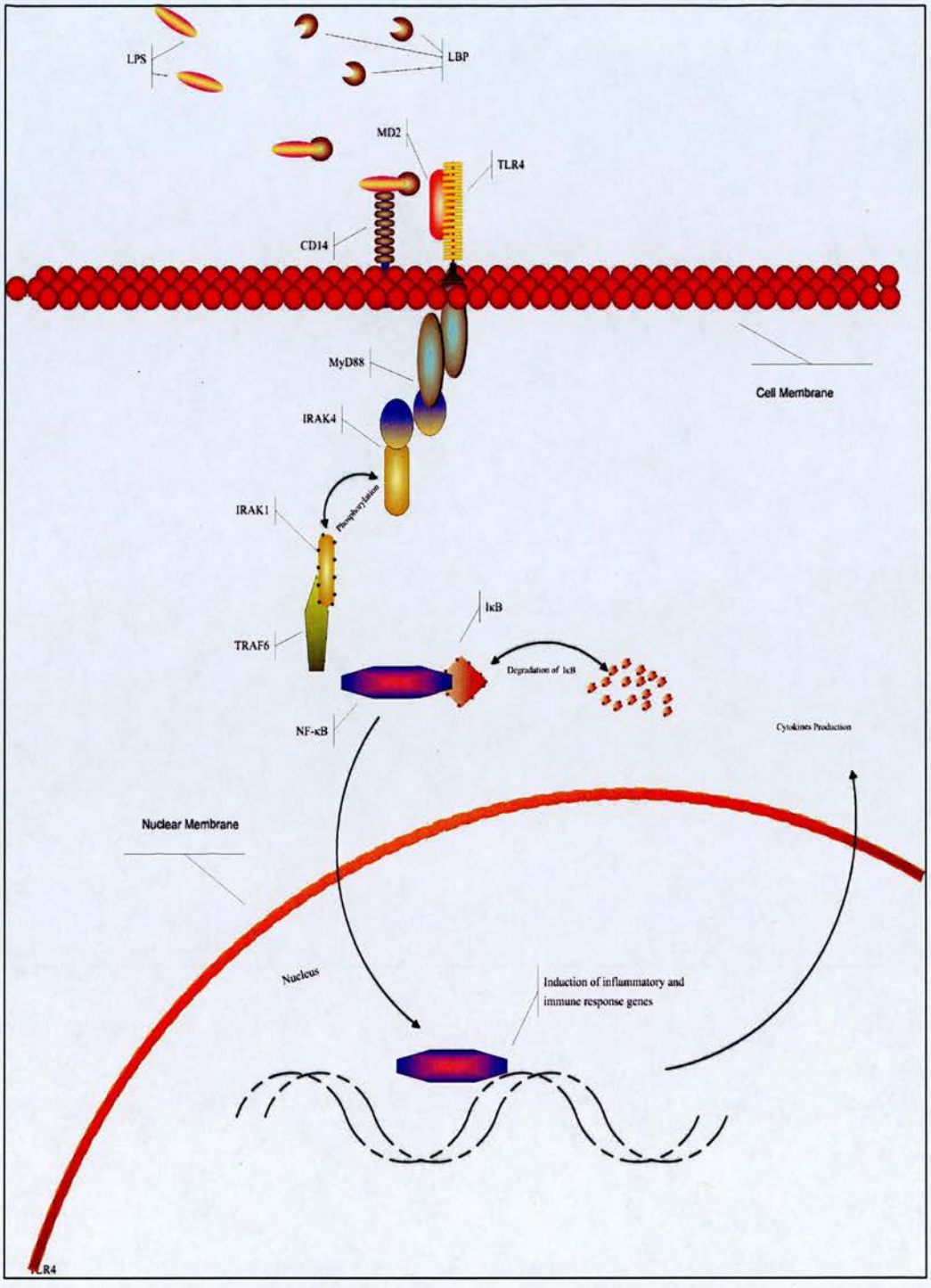

Figure 1.3 Common steps in inflammatory immune response

Natural antibodies plays a role in LPS clearance from the circulation as revealed by studies on recombinase-activating gene-2 (RAG-$2^{-/-}$)-deficient mice and Bruton's tyrosine kinase ($Btk^{-/-}$)-deficient mice, which lack serum antibodies and are known to be highly sensitive to endotoxin. In RAG-$2^{-/-}$ mice, both survival and clearance of endotoxin is improved when RAG-$2^{-/-}$ mice are inoculated with sera from normal mice. $Btk^{-/-}$ mice have reduced levels of IgG3 and IgM and demonstrated a significant increase in their capacity to clear endotoxin when they were reconstituted with purified normal mouse IgM (Reid *et al.,* 1997).

Another kind of innate response which may lead to a signalling pathway is the complement system which may have the capability to differentiate between LPSs of various bacteria. In the absence of specific antibodies, complement receptors present on monocytes/macrophages and dendritic cells could be stimulated via opsonization of bacteria. During scenarios of this nature, the classical complement pathway is stimulated by Lipid A and rough LPS, whereas the alternative pathway is stimulated by O-antigen polysaccharide. Moreover, CD14, complement receptor type 3 (CD11b/CD18) and TLR 4 have been shown to work together as LPS recognition and/or signalling receptors in macrophages (Caroff *et al.,* 2002). Nevertheless, it is revealed that monocytes and macrophages isolated from CD18-deficient patients show normal levels of TNF-α and IL-1, which indicating that CD18 in particular is not critical for LPS signalling (Wright *et al.,* 1990a). Moreover, it is also found that leukocytes which are treated by an antibody against CD18 do not show decreased level of TNF-α after challenging with LPS (Wright *et al.,* 1990b).

Although the binding between LPS and lipoprotein by itself contributes to the decreased LPS bioactivity, lipoprotein also participates as a key molecule in LPS elimination. It enhances the LPS clearance from the blood stream and delivery to the liver which is considered as the main organ responsible for the removal of endotoxin from the body (van Oosten *et al.*, 1998; Elsbach, 2000; Su, 2002). High-density lipoprotein (HDL) is known to act as one of main transport proteins of LPS in plasma. It has the capacity to bind fractions of LPS released when Gram-negative bacteria are incubated with plasma. Low-density lipoprotein (LDL) also has similar LPS binding properties. They both have a potential significant role in endotoxin

clearance since septic shock patients are found to have notably reduced plasma levels of HDL and LDL (Freudenberg *et al.,* 2001).

Another LPS-binding molecule is the bactericidal/permeability-increasing protein (BPI) which could differentiate between different LPS types and cause a decline in the LPS-dependent production of TNF-α and other cytokines in whole blood experiments. Furthermore, recombinant forms of BPI are demonstrated to protect animals against the fatal effects of LPS (Diks *et al.,* 2001).

The macrophage scavenger receptor (SR) class A type of receptors is another example of LPS-binding protein which is proposed to inhibit the inflammatory response by binding and eradicating LPS from the circulation. Indeed, the scavenger receptor conceals LPS from the host's immune system rather than initiating signalling. In parallel with this, the scavenger receptor is found to compete for LPS with the CD14 molecule. Moreover, studies show that the scavenger receptor play an essential role in ingestion of pathogenic structures for antigen presentation (Nicoletti *et al.,* 1999; Yamamoto *et al.,* 1999; Diks *et al.,* 2001).

The liver acts as a last barrier to prevent gastrointestinal bacteria and bacterial products, such as LPS, from entering the systemic blood stream. In this regard, Kupffer cells, and less significant endothelial cells and liver parenchymal cells, are the liver cells involved (van Oosten *et al.*, 1998). Experimental animals provide evidence of quick LPS clearance from the circulation within a few minutes of intravenous injection, most of it via the liver. The crucial function of the liver in LPS clearance can be verified in patients with liver failure, like cirrhosis, since they repeatedly suffer from endotoxaemia, not only that but the worse the liver failure the higher the endotoxaemia (Su, 2002). However, it is obvious that this approach does not contain cell signalling machinery in a way that leads to an inflammatory response (Heumann and Roger, 2002).

Acyloxyacyl hydrolase (AOAH) is a unique lipolytic leukocyte enzyme that has LPS-detoxification properties through its capacity to remove the secondary acyl chains of Lipid A (Munford and Hall, 1989; Erwin and Munford, 1990; Su, 2002). This partial deacylation mechanism eliminates the bioactivity of LPS, in addition to

creating such a reaction product which inhibits signalling by intact LPS in a competitive manner (Su, 2002).

1.3 The systemic inflammatory response syndrome

In healthy individuals, the normal plasma values of endotoxin are small and vary between 3 to 10 pg/ml (~0.1 EU/ml) as detected by standard *Limulus* amoebocyte lysate (LAL) assay techniques. On the contrary, systemic endotoxin levels reach high concentrations in patients suffering from severe sepsis or septic shock with plasma concentrations of more than 300 pg/ml (Alexander and Rietschel, 2001). It is observed that most patients in intensive care units have, on the day of admission, an elevated level of endotoxin which is related to both nonspecific illness severity, and infection of both Gram-negative and Gram-positive origin (Marshall, 2005). Furthermore, many investigators have shown that regardless of the origin of the infecting microorganism, the presence of endotoxin is frequent in the blood circulation in sepsis cases (Danner *et al.*, 1991; Hurley, 1994; Hurley, 1995a; Opal *et al.*, 1999). On the other hand, several studies that involved intensive care patients with sepsis or septic shock found that endotoxaemia was established in only 20 to 40% of those patients. Although these result were linked to LAL-positivity, they give an indication that endotoxin is not always detected in the blood of septic patients (Cohen, 2000). Furthermore, it is also difficult to conclude that whenever endotoxaemia exist it must indicate the presence of Gram-negative infection. Nevertheless, it is noticeable that endotoxaemia and Gram-negative bacteraemia are not synonymous; patients may suffer from bacteraemia due to either Gram-negative or Gram-positive bacteria without any indication for the presence of free endotoxin in the blood. Similarly, endotoxaemic patients are not inevitably bacteraemic nor have an infection focus elsewhere. So, as a conclusion, endotoxaemia by itself is not linked to an identified Gram-negative infection or bacteraemia (Cohen, 2000).

Of special interest, the relationship between high mortality and the presence of endotoxin is not readily explicable in older patients. Although endotoxin challenge studies in aged animals demonstrated that they do not tolerate endotoxin as well as younger animals. This may, in some way, be due to the variation in the metabolic

response to endotoxin among different age groups which eventually effect sensitivity to endotoxin (Opal *et al.,* 1999).

In a relatively homogeneous population, like patients with meningococcal disease, it is observed that patients with meningococcal meningitis have both an absent or low levels of endotoxin in the blood and low mortality rate. However, meningococcaemia endotoxaemia patients have the opposite situation with a positive quantitative association between the level of endotoxin in the blood and the outcome (Brandtzaeg *et al.,* 1989). This relationship is by far much more complicated to disclose in a heterogeneous ICU population. Nevertheless, analysis of 11 studies concluded that the presence of both Gram-negative bacteraemia and endotoxaemia did define a subcategory of patients at higher risk of dying (Hurley, 1995b). In light of this notion, some investigators introduced the idea of a "sepsis score" by which they consider the levels of endotoxin, TNF-α, IL-1 and IL-6. These studies found a significant correspondence between the higher scores and poor outcome (Casey *et al.,* 1993). This may indicate that endotoxaemia is not the only indicator for mortality rate in ICU patients. However, several studies support the observation that patients with endotoxaemia, and in particular those with elevated levels of endotoxin, are expected to be more severely ill (Cohen, 2000).

It is clear now that Gram-positive bacteria are one of the main causes of sepsis and septic shock (Horn *et al.,* 2000). This suggests that new players, such as exotoxins, peptidoglycans, lipoteichoic acid and prokaryotic DNA, rather than endotoxin are involved (Heumann *et al.,* 1994; Dziarski *et al.,* 1998; Schromm *et al.,* 1999). However a recent study demonstrated that endotoxin was detected in the patients who have no microbial growth on their sample culture at levels almost similar to those patients who have reported infection from intra-abdominal, lung and blood sites (Marshall *et al.,* 2004). Such study supports the translocation hypothesis of endotoxin from the gastrointestinal tract which is considered to contain approximate 25 grams of endotoxin (van Deventer *et al.,* 1988; Doig *et al.,* 1998).

From this site, Gram-negative bacteria and their endogenous endotoxin are thought to penetrate the disrupted permeability barrier of the colon due to the inflammation-mediated mechanisms induced by even Gram-positive sepsis (Doig *et al.,* 1998; Horn

et al., 2000). Moreover, local hypoperfusion and mucosal ischaemia are assumed to contribute to the endotoxin translocation from the intestine to the blood circulation (Rocke *et al.*, 1987; van Deventer *et al.*, 1988; Hurley, 1995a). Additionally, it is reported that sepsis and systemic inflammatory response syndrome are complications after cardiovascular or vascular surgery because of the translocation of endotoxin from the intestine (Martinez-Pellus *et al.,* 1997; Sugita *et al.,* 1998). Moreover, different illnesses or even invasive procedures, varying from multiple trauma, ankle fracture and colonoscopy, have been linked to systemic endotoxaemia. Such examples support the hypothesis that the endotoxin is mainly derived from the gut (Marshall, 2005).

Septic shock patients who have circulating endotoxin are found to suffer from severe physiological changes and high mortality rate (Danner *et al.,* 1991). Symptoms of sepsis in human are similar in many ways to the pathophysiological events that happened as a result of the administration of endotoxin to humans and experimental animals (Horn *et al.*, 2000; Hurley, 2003). In addition to fever, endotoxin can contribute to multiple biological effects like leukocytosis, platelet aggregation, thrombocytopenia, leukopenia, tachycardia, tachypnea, hypotension, disseminated intravascular coagulation, and multi-organ failure (Hurley, 1995a; Schletter *et al.*, 1995b). Apparently, sepsis is an outcome of incapacity in the immune system to overcome an immense bacterial load which outweigh the inhibition mechanisms that control inflammation (Decker, 2004).

For almost a century, sepsis was defined as the systemic host response to an infection. While the original definition of the sepsis was supposed to be linked to the existence of bacteria in the blood (bacteraemia). Both of the terms "sepsis" and "septicaemia" were repeatedly interchanged in their usage in the clinical situation. Some investigators introduced another simple definition for the sepsis syndrome that was a specific clinical manifestations including a known source of infection (Riedemann *et al.,* 2003).

Sepsis is linked to several terms and nomenclature, which indicate the level of complexity to distinguish between many symptoms of inflammatory responses that are assumed to be identical regardless of their causes. In 1991, the American College

of Chest Physicians and the American Society of Critical Care Medicine published a new scheme of definitions for what they termed systemic inflammatory response syndrome (SIRS) and sepsis. They tend to concern the way the cases were diagnosis and the treatment method. Critically ill patients with different underlying illneses such as trauma, burns, pancreatitis and infection, were shown to have a group of inflammatory response variables including leucocytosis or severe leucopoenia, hyperthermia or hypothermia, tachycardia and tachypnoea which are all termed together as systemic inflammatory response syndrome (SIRS). This definition highlights the magnitude of the inflammatory process itself, whether it is accompanied by infection or not. Therefore, SIRS that is linked with suspected or confirmed infection is designated by another term, sepsis. When evidence of organ failure is detected, the patient transfers to another level which is called severe sepsis in which several other signs are observed, such as hypoxaemia, oliguria, lactic acidosis or altered cerebral function. Moreover, septic shock is reached when a case of severe sepsis is accompanied by hypotension with systolic blood pressure less than 90 mmHg even with sufficient fluid resuscitation. Additionally, sepsis and SIRS may get worse if the failure of two or more organs are detected which is termed a multiple organ failure (MOF) (Paterson and Webster, 2000). MOF is considered to be the eventual cause of death in sepsis patients. Typically, patients will primary suffer from a single organ failure like respiratory failure needing mechanical ventilation and then if not treated, it will develop a multiple organ failure. There is a close association between the severity of organ failure on admission day to an intensive care unit and the likelihood of survival, and between the number of failed organs and the threat of death. Four or five failed organs translates to a mortality of more than 90%, regardless of treatment. The organ failure pathogenesis is not yet fully understood due to the influence of multiple risk factors such as tissue hypoperfusion and hypoxia (Cohen, 2002).

However, there are two ways to interpret these kinds of inflammatory response complications. On the one hand, it is proposed that severe infection leads to a reduced capacity of the immune system to generate an effective antimicrobial response which ultimately results in prevalence of paralysis of the immune system. On the other hand, it is assumed that specific microbial components, such as

endotoxin, stimulate a powerful immune reaction that leads to an excessive production of immune mediators that eventually gives rise to multiple organ failure (Pfeffer, 2003).

From 1979 through to 1987, Gram-negative bacteria were considered to be the leading organisms causing sepsis. Nevertheless, in 2002 Gram-positive bacteria were shown to be the most important cause of sepsis with 52.1%, followed by Gram-negative bacteria with 37.6%, polymicrobial infections with 4.7%, anaerobes with 1.0% and fungi with 4.6%. Organ failure had a deteriorating effect on the overall outcome since the mortality rate among patients without organ failure is about 15% , while it is 70% among patients with three or more organ failures (Martin *et al.,* 2003; Riedemann *et al.,* 2003). Nevertheless, other recent studies have demonstrated that Gram-negative bacteria accounted for about 60% of sepsis cases, where Gram-positive were responsible for the rest (Angus *et al.,* 2001; Alberti *et al.,* 2002). In another regard, the immunopathogenesis of septic shock caused by Gram-positive bacteria is considerably different from that observed in Gram-negative sepsis.(Horn *et al.,* 2000). Needless to say that both of them are different in the way they initiate disease. Since Gram-positive bacteria usually depend on the production of powerful exotoxins (sometimes superantigens) while Gram-negative bacteria depend on the production of endotoxin. Moreover, they also differ in the origin site from which sepsis arose. Gram-positive sepsis often arise from skin, wounds, soft-tissue structures, and catheter sites whereas Gram-negative sepsis often arise from enteric or genitourinary sources (Bone, 1994). Morever, there are differences in both the nature and timing of the responses. Endotoxin induces a rapid response of proinflammatory cytokines, primarily TNF-alpha, IL-1, IL-6, and IL-8. On the contrary, the Gram-positive toxins induces a typical Th-1-type cytokine response, dominated by TNF-beta and interferon-gamma. TNF-alpha and IL-1 were produced but less than in response to endotoxin. Furthermore, the rhythm of the cytokine response to Gram-positive toxins is different from the Gram-negative response, with peak response delayed to perhaps 50-75 hrs after the challenge, in contrast to the 1-5 hrs response to endotoxin (Andersson *et al.*, 1992). Another study showed an obvious difference in responses between Gram-negative endotoxin and Gram-positive exotoxins when they both challenge human peripheral blood mononuclear

cells. IL-8 was the most abundant cytokine produced in response to the exotoxins, while endotoxin was most active in inducing IL-1, IL-6, and TNF-alpha (Muller-Alouf *et al.*, 1994).

Analysis of discharge data on approximately 750 million hospitalization in the United States over a 22-year period recognized 10,319,418 cases of sepsis which represent 1.3% of all hospitalizations. An annual increase in the incidence of sepsis is noticed from about 164,000 cases (82.7 per 100,000 population) in 1979 to nearly 660,000 cases (240.4 per 100,000 population) in 2000. In the USA and Europe, the estimated affected patients who suffer from sepsis vary between 400,000 to 500,000 on an annual basis (Guha and Mackman, 2001). Another study, derived from the US data, revealed that the cases of severe sepsis that occurred in 1995 only were 751,000, from which the observed mortality rate was 28.6%, and this turns into about 215,000 deaths. As one of the leading causes of death in the US since 1950, sepsis showed the largest increase in death rate which increased 38-fold, from 0.3 in 1950, to 11.5 in 2000, per 100,000 (Russo and Johnson, 2003). Moreover, sepsis is considered as an outcome related to the risk factors such as prolonged stay both in intensive care unit (ICU) and in hospital (Granja *et al.,* 2004). However, a recent epidemiological study from North America indicated that the incidence of sepsis was about 3.0 cases per 1,000 of US population, which means 750,000 cases annually. The overall mortality was approximately 40%, varying from 30% in the elder patients to 50% or higher in patients suffering from severe symptoms of septic shock (Angus *et al.,* 2001). Another study linked the mortality rate of 20-50% to septic shock syndrome which results in annual numbers of 100,000 deaths in the USA (Schletter *et al.,* 1995b). A third conservative study showed that the annual resultant deaths from sepsis were between 20,000 –50,000 only in the United States (Bone, 1991). Another study estimated that approximately 700,000 people suffered from sepsis annually and the annually resultant deaths from sepsis are between 100,000 to 210,000 in the United States (Nogare, 1991; Riedemann *et al.*, 2003). In view of that, sepsis results in an estimated $16.7 billion per year as a costs to the US health care system (Riedemann *et al.,* 2003). Moreover, estimated cost of sepsis patient care is about as $50,000 per patient. Sepsis is considered to be the second highest reason of death among patients in non-coronary intensive care units and the tenth top reason of

death in general in the United States. Furthermore, sepsis significantly contributes to the low-quality life of the survival patients (Martin *et al.,* 2003). In general, septic patients stay in hospital for prolonged periods and they rarely depart the ICU before 2–3 weeks (Riedemann *et al.,* 2003).

1.4 Aims of thesis

It becomes widely recognizable that different LPSs from different bacteria may interact, signal in different way. It was, therefore, reasonable to choose structurally and functionally different LPSs from different bacterial species that represent what may happen inside the human host when bacteria or their LPS reach the blood stream for instance in the case of sepsis. It is also rational to investigate how different methods of extraction and purification will affect the signalling activity of LPS quantitatively and qualitatively. Accordingly, three main aims for this study were investigated

1) Investigating the effects of different LPS extraction methods on the activity of different LPS preparations from four different bacteria (*Escherichia coli* O18K-, *Pseudomonas aeruginosa* Pa-O1, *Bacteroides fragilis* NCTC 9343 and *Rhodobacter sphaeroides* NCIMB 8253) and their ability to produce proinflammatory cytokines (TNF-α and IL-1β).
2) Investigating the effect of mixing different LPS preparations extracted by different methods from three different bacteria (*E. coli* O18K-, *B. fragilis* NCTC 9343 and *R. sphaeroides* NCIMB 8253) on producing proinflammatory cytokines.
3) Investigating how LPS repurification method, different LPS extraction methods of *Bacteroides fragilis* together with different heat killed *B. fragilis* populations might affect the toll like receptor (TLR) specificity.

CHAPTER 2: MATERIAL AND METHODS

2.1 General chemicals

All chemicals used were of Analar grade, unless mentioned otherwise. Aqueous solutions used in all lipopolysaccharide studies were made up in pyrogen free water (PFW), others were made up in distilled water (Millipore, Billerica, MA, USA). Phosphate buffered saline (PBS) was prepared in all experiments by dissolving one tablet of PBS (Oxoid, Basingstoke, UK) per 100ml of PFW and autoclaved prior use. All other autoclavable chemicals were autoclaved before use.

2.2 Media for bacterial growth

The following media were used throughout this study.

2.2.1 Nutrient broth medium (NB)

Filter-sterilized nutrient broth was purchased from Gibco (Paisley, UK) and used for aerobic growth of *Escherichia coli* and *Pseudomonas aeruginosa* strains.

2.2.2 Proteose Peptone Yeast Extract Medium (PPY medium)

PPY medium was used for an anaerobic growth of *Bacteroides fragilis* strains. Proteose peptone (Oxoid) 20g, yeast extract (Oxoid) 10g, NaCl 5g, cysteine HCl (3.75% w/v solution) 20ml, solution carbonate (2% w/v solution) 20ml, haemin(250µg/ml)/menadione (50µg/ml) were added to 940ml of PFW, autoclaved at 121°C for 15min and stored at 4°C.

2.2.3 R8AH medium (modified ATCC 550 liquid medium)

Rhodobacter sphaeroides was grown on R8AH medium which is recommended by the American Type Culture Collection (ATCC) according to the original work of Drews (1965) and Weckesser *et al.*(1972) with a slight modification.

Trace element requirements of this medium were prepared separately at 100x the trace element concentration as indicated in Table 2.1

Table 2.1 List of trace element used for R8AH medium

Trace element	Concentration
Manganese chloride.$4H_2O$ ($MnCl_2.4H_2O$)	20mg to 100ml PFW
Boric acid (H_3BO_3)	10mg to 100ml PFW
Copper sulfate ($CuSO_4.5H_2O$)	10mg to 100ml PFW
Ammonium heptamolybdate ($(NH4)_6Mo_7O_{24}.4H_2O$)	20mg to 100ml PFW
Zinc sulfate($ZnSO_4$)	10mg to 100ml PFW

Then, 300mg of ammonium ferric citrate, 63.7mg EDTA.$Na_2.2H_2O$ and $CaCl_2.2H_2O$ were dissolved in 95ml of PFW and 1ml of each of the 100x trace elements (Table2.1) were added. Vitamin solution at 100x concentration was prepared by adding 8mg to 10ml PFW. 20mg nicotinic acid, 20mg nicotinamide and 40mg thiamine HCl were dissolved in 99ml PFW and 1ml of 100x biotin solution was then added. All solutions were stored in 4°C.

Complete medium was prepared by dissolving malic acid in 900ml of PFW which then neutralized with 1M NaOH. The remaining ingredients were added as stated in Table 2.2. Finally the medium was autoclaved at 121°C for 15min and stored at 4°C.

Table2.2 Ingredients of R8AH complete medium

Ingredients	Weight or volume
Malic acid ($C_4H_6O_5$)	2.5g
Yeast extract (BD 212750)	1.0g
Ammonium sulfate ($(NH_4)_2SO_4$)	1.25g
Magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$)	0.2g
Calcium chloride ($CaCl_2$)	0.07g
Ammonium ferric citrate ($C_6H_8O_7.nFe.nH_3N$)	0.01g
Ethylenediaminetetraacetic acid (EDTA) ($C_{10}H_{16}N_2O_8$)	0.02g
Potassium dihydrogen phosphate (KH_2PO_4)	0.6g
Dipotassium phosphate (K_2HPO_4)	0.9g
Trace elements (See above)	1.0ml
Vitamin solution (See above)	7.5ml
PFW to	1000ml

2.3 KIT-systems

The KIT-system used in this study are listed in Table 2.3 .

Table 2.3 KIT-systems used in this study

KIT-system	Source
DuoSet® ELISA Development system for measuring human TNF-α/TNFSFIA (DY210)	R&D, Abingdon,UK
DuoSet® ELISA Development system for measuring human IL-β/IL-1F2 (DY201)	R&D, Abingdon,UK
Pyrochrome LAL kit	Associates of Cape Cod, Falmouth, MA, USA

2.4 Cell lines

The cell lines used in this study are listed in Table 2.4 .

Table 2.4 Cell lines used in all experiments

Cell line	Origin	Source
Human embryonic kidney cell cultures (HEK-293)	Transformed from normal human epithelial embryonic kidney cells with sheared adenovirus 5 DNA, Graham *et al.*(1977)	Kind gift from Clett Erridge (Strathclyde University, Glasgow,UK) originally from (ECACC, Salisbury, U.K.)
Human acute monocytic leukemia cell line (THP-1)	Derived from human male 1 year old, Tsuchiya *et al.*(1980)	European Collection of Animal Cell Cultures (ECACC, Salisbury, U.K.) ECACC No: 88081201
Mouse C3H/An connective tissue cell line (L929)	Derived from normal subcutaneous areolar and adipose tissue of 100 day old male C3H/An mouse, Earle(1943)	European Collection of Animal Cell Cultures (ECACC, Salisbury, U.K.) ECACC No: 85011425

2.5 Maintenance of cell lines

2.5.1 L929 cell line

Cells were routinely sub-cultured using DMEM/10%FCS which was prepared by adding 50ml of sterile filtered foetal calf serum (FCS, Sigma), 5ml of penicillin streptomycin and glutamine (PSG, Gibco) to 500ml of DMEM medium (Sigma). The same medium formula was used for the adhering stage before TNF-α bioassay except 25ml of FCS was added, (DMEM/5%FCS). For the TNF-α bioassay 50µl of Actinomycin D (Stock of 5mg/ml in DMSO, Sigma) was added to the DMEM/5%FCS (DMEM/5%FCS/ActD).

As an adherent cell line, L929 was washed using sterile PBS and disaggregated by a solution which was prepared by adding 2ml of Trypsin-EDTA (Gibco) to 20ml of sterile PBS.

10ml of freezing medium for L929 cell line contained 1 ml of dimethylsulphoxide DMSO (Sigma) and 9 ml of FCS.

2.5.2 THP-1 cell line

Cells were grown in a start-up culture contained 30ml of RPMI-1640 medium (Sigma), 6ml of FCS, 3ml of 100mM autoclaved 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, (HEPES) (Gibco) (RPMI/20%FCS/HEPES). For routine growth, cells were sub-cultured using (RPMI/10%FCS/PS+G) which was prepared by adding 50ml of FCS, 5ml of penicillin streptomycin and 15ml of 200mM Glutamine (Sigma) to 500ml of RPMI medium. For the differentiation stage, cells were grown using (RPMI/PSG) which was prepared by adding 5ml of PSG to 500ml of RPMI medium, cells were then stimulated by adding 2ml of 100nM Vitamin D3 (Sigma) to 20ml of the cell suspension. To maintain cell growth after differentiation stage, cells were grown in (RPMI/10%FCS/PSG) which was prepared by adding 50ml of FCS and 5ml of PSG to 500ml of RPMI medium. 10ml of freezing medium for THP-1 cell line contained 3 ml of sterile glycerol (Sigma) and 7 ml of FCS.

2.5.3 HEK-293

Cells were routinely sub-cultured using DMEM/10%FCS as mentioned above. Cells were grown in DMEM medium plus PSG during transfection stage (DMEM0). For sample stimulation stage, cells were grown using (DMEM/1%FCS) which was prepared by adding 5ml FCS and 5ml of PSG to 500ml of DMEM.

2.5.4 Determination of cell viability

Viability of cell lines was determined using trypan blue dye (Sigma) assay which is based on the exclusion of the dye from viable cells (Mishell and Shiigi, 1980). One volume of cells harvested at 100g for 5 min were added to an equal volume of trypan blue dye and mixed well. Cells were then immediately observed under a microscope using a dual-chamber haemocytometer. Stained and unstained cells were recorded separately and the viable cell ratio was calculated according to the following equation:

(Number of unstained cells/Total number of cells) × 100 = Percent Viable Cells.

2.6 Bacterial strains

The bacterial strains which used during this study are listed in Table 2.5 .

Table 2.5 Bacterial strains used in all experiments

Species	Strain
Escherichia coli	O18K$^-$ (MPRL 1275)
Pseudomonas aeruginosa	PAO1 (MPRL 664)
Bacteroides fragilis	NCTC 9343 (MPRL 1669)
Rhodobacter sphaeroides	NCIMB 8253(MPRL 4788)
Porphyromonas gingivalis	NCTC 11834

2.7 Reagents

The lipopolysaccharide preparations used in this study are listed in Table 2.6 .

2.7.1 Lipopolysaccharides

Table 2.6 Lipopolysaccharides used in all experiments

Species	Extraction Method	Abbrev.	Extracted by
E. coli	Triton /Proteinase K	EC1	M. Al-hawi
E. coli	Triton	EC2	M. Al-hawi
E. coli	Aqueous phenol	EC3	M. Al-hawi
E. coli	Boiling H_2O /Proteinase K	EC4	M. Al-hawi
P. aeruginosa	Triton	PA1	M. Al-hawi
P. aeruginosa	Triton /Proteinase K	PA2	M. Al-hawi
P. aeruginosa	Aqueous phenol	PA3	M. Al-hawi
P. aeruginosa	Boiling H_2O /Proteinase K	PA4	M. Al-hawi
B. fragilis	Triton	BF1	M. Al-hawi
B. fragilis	Triton /Proteinase K	BF2	M. Al-hawi
B. fragilis	PCP	BF3	M. Al-hawi
B. fragilis	Boiling H_2O /Proteinase K	BF4	M. Al-hawi
B. fragilis	Aqueous phenol	BF5	M. Al-hawi
R. sphaeroides	Aqueous phenol	RS1	M. Al-hawi
R. sphaeroides	PCP	RS2	R. Brown
R. sphaeroides	PCP	RS3	R. Brown
R. sphaeroides	PCP	RS4	R. Brown
P. gingivalis	Aqueous phenol	PG	Gift from C. Erridge

2.7.2 Heat killed bacteria

The heat killed bacteria preparations used in this study are listed in Table 2.6 .

Table 2.7 Heat killed bacteria used in all experiments

Species	Description	Abbrev.	Source
B. fragilis	Large capsule population	HKLC	Gift from Prof Sheila Patrick (Queen's University Belfast, Belfast,UK)
B. fragilis	Small capsule population	HKSC	
B. fragilis	Electron dense layer population	HKEDL	

2.8 Bacterial growth conditions

The purity of all cultures was checked by performing Gram stain films and a 48h purity plate.

2.8.1 Anaerobic growth

Anaerobic conditions (10%H_2, 10%Co_2 + 80%N_2) were used to culture *B. fragilis* species in PPY broth medium at 37°C overnight in an anaerobic work station. Bacterial cells were then harvested by centrifugation at 10,000g for 15min at 4°C then washed once with wash buffer (depended on extraction method), centrifuged again at 10,000g for 10min at 4°C. Finally the bacterial pellets are washed for the last time with PFW with same centrifugation settings. The bacterial pellet was frozen at -20°C, freeze dried and weighed.

2.8.2 Aerobic growth

E. coli and *P. aeruginosa* were grown aerobically in NB medium at 37°C overnight, shaken at 150 rpm. Bacterial species of *R. sphaeroides* was grown in ATCC 550 broth medium at 30°C overnight in the dark and shaken at 150 rpm. Bacterial cells were then harvested by centrifugation at 10,000g for 15min at 4°C then washed once with wash buffer (depended on extraction method), centrifuged again at 10,000g for 10min at 4°C. Finally the bacterial pellets are washed for the last time with PFW with same centrifugation settings. The bacterial pellet was frozen at -20°C, freeze dried and weighed.

2.9 Extraction of lipopolysaccharide

Different preparations of LPS have been used in this study. LPSs were obtained by extraction of lyophilized bacterial cell by different extraction methods.

2.9.1 Aqueous Phenol (AP) method

Wash buffer used prior to this method for harvesting bacteria was prepared by dissolving 1.48g sodium dihydrogen phosphate ($NaH_2PO_4.2H_2O$), 5.749g disodium hydrogen orthophosphate (Na_2HPO_4) and 8.77g sodium chloride (NaCl) in 1 litre of PFW.

LPSs were extracted from *E. coli*, *P. aeruginosa*, *B. fragilis* and *R. sphaeroides* using the aqueous phenol method as described by Westphal and Luderitz (1954). Pre-weighed, freeze-dried bacteria were ground-up and suspended to 5% w/v in PFW. The preparation was heated in a waterbath at 67°C and an equal volume of preheated (67°C) 90% w/w phenol solution was added. The well-mixed solution was incubated at 67°C for 15min and then cooled in an ice bath to allow phases to separate. The solution was centrifuged at 10,000g for 15min at 4°C. The resulting clear upper aqueous layer containing LPS material was then carefully transferred to dialysis tubing (Medical International Ltd, London). The centrigugation step was repeated and the pooled aqueous layers were dialysed against tap water overnight. Dialysed opalescent supernatant was concentrated under vacuum on a rotary evaporator then ultracentrifuged at 100,000g for 3h at 4°C. The resulting pellet was resuspended in PFW and ultracentrifuged again at the same previous settings. Finally, the LPS pellet was resuspended in PFW and freeze-dried then stored at -20°C.

2.9.2 Phenol/chloroform/petroleum spirit (PCP) method

Wash buffer used prior to this method was the same as for aqueous phenol method. PCP reagent was prepared by adding 90% w/w aqueous phenol, chloroform and petroleum spirit in the ratio of 2:5:8 by volume. Diethyl ether was added to acetone in the ratio of 1:5 by volume.

PCP method was implemented according to the work of Galanos *et al.*(1969) in addition to the further procedure of using diethyl ether/acetone mixture proposed by Qureshi *et al.*(1982) for extraction of rough LPS from *B. fragilis*. Pre-weighed, freeze-dried bacteria were ground-up and suspended as one part of bacterial pellet in at least 12 parts of PCP extraction mixture until a fine suspension was obtained. This preparations was centrifuged twice at 10,000g for 15min at 4°C. The resulting supernatant was filtered through Whatman No. 1 filter paper. The bacterial residue was extracted once more as above and the filtered supernatant was added to the first one. The filtered supernatant was then rotary evaporated to be followed by mixing of one volume of LPS-containing phenol solution with six volume of diethyl ether/acetone mixture at room temperature for 1h. Next centrifugation step was carried out at 5000g for 10min at room temperature. The resultant pellet was resuspended in the previous mixture and centrifuged for three times. LPS was then dried in a vacuum desiccator and resuspended again in PFW to be ultracentrifuged for the last time at 100,000g for 4h at 4°C. Finally, the LPS pellet was resuspended in PFW and freeze dried then stored at -20°C.

2.9.3 Triton/magnesium chloride (TM/TMP) method

Wash buffer used prior to this method for harvesting bacteria was prepared by dissolving 7.57g Tris (hydroxymethyl) methylamine ($NH_2C(CH_2OH)_3$) in 1000ml PFW, pH was then adjusted to 6.8 with 1M HCl and 8.77g sodium chloride (NaCl) was then added. Solutions for different methods were prepared as outlined in Table 2.8.

Table 2.8 Solutions used in Triton/magnesium chloride method

Solution	Components	Preparation
TS1	100mM Tris/HCl, pH 8.0	6.055g Tris in 500ml PFW
TS2	1M $MgCl_2.6H_20$	40.662g magnesium chloride in 200ml PFW
TS3	0.5M $MgCl_2.6H_20$	10.166g magnesium chloride in 100ml PFW
TS4	8% (v/v) Triton X-100	40ml Triton X-100 in 460ml PFW
TS5	0.2M EDTA, pH 8.0	18.613g EDTA in 250ml PFW
TS6	2M NaCl	29.220g NaCl in 250ml PFW
TS7	10mM Tris/HCl/10mM $MgCl_2$, pH 8.0	100ml of TS1 and 10ml TS2 to 890ml PFW

LPSs were extracted from *E. coli*, *P. aeruginosa* and *B. fragilis* using the Triton/magnesium chloride method with (TMP) and without (TM) Proteinase K treatment according to the work of Uchida and Mizushima (1987). This method consists of three parts. At the end of the first part an insoluble form of LPS was collected as a pellet after one gram freeze-dried bacteria was ground-up and suspended in 22ml PFW followed by successively adding 4ml TS1, 4ml TS3, 10ml TS4 and 10ml ethanol (EtOH) . This mixture then boiled in a vigorously boiling bath for 20min, cooled, centrifuged at 20,000g for 20min. Then pellet was resuspended in 40ml TS7 and finally centrifuged at 100,000g for 90 min at room temperature.

In the second part, the washed precipitate was mixed well with sequence adding of 10ml PFW, 10ml TS5, 10ml TS6 and 10ml TS4 , incubated in a shaking waterbath at 37°C for 60min then centrifuged at 20,000g for 25min at room temperature. The resulting supernatant containing the LPS was centrifuged again to ensure complete removal of insoluble materials.

In part three, 4ml TS2 was added to the supernatant, to precipitate the LPS, and the mixture incubated at 37°C for 60min. The LPS was collected by ultra-centrifugation at 100,000g for 90min at room temperature. At this stage, the optional Proteinase K treatment was implemented. After resuspending the resulting clear precipitate in PFW, the solution was treated with Proteinase K (20µg/ml) at 37°C for 2h.

Then, the Proteinase K - or non treated material were washed once in 40ml TS7, ultra-centrifuged at 100,000g for 90min at room temperature. Finally, the LPS pellet was resuspended in PFW and freeze dried then stored at -20°C.

2.9.4 Boiling water/ Proteinase K treatment (BWP) method

Wash buffer containing 20mM Tris-HCl, 0.15M NaCl, 10mM $MgCl_2$ at pH 7.4 was used prior to this method for harvesting bacteria according to the method of Yoshimura *et al.*(1984).

LPSs were extracted from *E. coli*, *P. aeruginosa* and *B. fragilis* using a rapid boiling water/ Proteinase K treatment method as proposed by Eidhin and Mouton (1993). Freeze-dried bacteria of 25mg were suspended in 1ml of PFW and boiled in a water bath for 15min with vortexing at 5min intervals. The mixture was then centrifuged at 12000g for 5min. The resulting supernatant was collected, treated with Proteinase K (1mg/50µl PFW) and incubated at 60°C for 1h before being transferred to a boiling water bath for 5min to precipitate any residual Proteinase K and centrifuged as before. The resulting supernatant was then subjected to an overnight dialysis against water and subjected to a second round of precipitation and centrifugation. Finally, the resulting supernatant was freeze-dried and stored at -20°C.

2.10 Depigmentation of *R. sphaeroides* method

Prior to LPS extraction, freeze-dried *R. sphaeroides* were subjected to a depigmentation procedure to remove red pigment which is known to interfere with the extraction of LPS according to the work of Strittmatter *et al.*(1983). Freeze-dried cells (2.8g) were suspended in 200ml of PFW and centrifuged at 10,000g for 10min at 4°C. The pink freeze-dried cells turned green after being treated three times with 100ml butan-1-ol containing 1% acetic acid and centrifuged at 10,000g for 10min at 4°C. The resulting pellet was then treated three times with 100ml EtOH and centrifuged at 10,000g for 10min at 4°C when it turned brown in colour.

Bacterial cells then turned orange-red after being treated three times with 100ml acetone and centrifuged at 10,000g for 10min at 4°C. The colourless pellet was achieved after treated bacterial cells were washed three times with 100ml ether, centrifuged at 10,000g for 10min at 4°C and finally dried in-vacuum. LPS was then extracted by the methods outlined above.

2.11 Re-purification of LPS

All LPSs extracted by the different methods were subjected to repurification to eliminate possible protein contaminants, that are active in signalling via TLR2 on C3H/HeJ macrophages (Manthey *et al.*, 1994), according to the work of Manthey and Vogel (1994) that was detailed further by Hirschfeld *et al.*(2000). LPS (5mg) was dissolved in 1ml PFW containing 0.2% triethylamine (TEA) at room temperature. A 500µl volume of this preparation was added to 100µl of 3% sodium deoxycholate solution and briefly mixed. Water-saturated phenol (600µl:WSP) solution was then added to the mixture which was intermittently vortexed for 5min at room temperature. The phases were allowed to separate for a further 5min at room temperature before being placed in an ice-bath for 5min and then centrifuged at 10,000g for 2min at 4°C. After the top aqueous layer was transferred to another tube, the lower phenol phase was re-extracted with 600µl of 0.2% TEA/0.5% sodium deoxycholate solution as above. Again the top aqueous layer was transferred and pooled with the other top aqueous layer previously collected. The aqueous phases were re-extracted with 600µl of WSP as above. The top aqueous phase was collected, and adjusted to 75% EtOH/ 30mM sodium acetate, allowed to precipitate at -20°C for 1h and then centrifuged at 10,000g for 10min at 4°C. The resulting precipitate was washed in 1ml cold 100% EtOH and the precipitate dried at room temperature. Almost one hundred percent of LPS was assumed to be recovered from this procedure.

To enhance LPS solubility, triethylamine (TEA) was added to all crude or repurified LPS preparations as 1mg of LPS/1ml of PFW/1µl of TEA and frozen at -20 °C.

2.12 LPS and protein analysis

2.12.1 Preparation samples for polyacrylamide gel electrophoresis (PAGE)

LPS samples extracted by the four different methods were suspended in PFW at 1mg/ml. Just prior to loading onto gels samples were mixed with an equal volume of double strength PAGE sample buffer, boiled for 3min and then allowed to cool to room temperature. Double strength PAGE sample buffer (0.125M Tris/HCl pH6.8, 20% glycerol, 2% 2-mercaptoethanol, 0.002% bromophenol blue) was prepared by dissolving 1.514g Tris, 25.2g glycerol, 2ml 2-mercaptoethanol, 4ml bromophenol blue in 100ml PFW.

2.12.2 PAGE

Analysis of LPS preparations was performed using the buffer system proposed by Laemmli (1970). PAGE of LPS samples was performed omitting sodium dodecyl sulfate from both separating and stacking buffers (non-SDS PAGE). Double strength separating gel buffer (0.75M Tris/HCl pH8.8) was prepared by dissolving 22.72g Tris in 250ml PFW. Double strength stacking gel buffer (0.25M Tris/HCl pH8.8) was prepared by dissolving 7.571g Tris in 250ml PFW. Both separating and stacking gels were prepared as outlined in Table 2.9 and deaerated under vacuum prior to adding TEMED (NNN'N-tetramethyl-1,2-diaminoethane, BDH) and ammonium persulphate (BDH). A 40% w/v aqueous acrylamide/ methylenebisaccrylamide solution was prepared by dissolving 100g acrylamide, 2.7g methylenebisacrylamide in 250ml PFW.

The separating gel was loaded between two assembled glass plates which had been cleaned with methylated spirit and sealed with molten Vaseline. The gel was covered with a thin layer of water-saturated butan-2-ol (BDH) and allowed to polymerize. After the butan-2-ol was removed, a 4% stacking gel was poured onto the separating gel. A comb was fitted and the gel allowed to polymerize. After the comb was removed, the gel was placed into an electrophoresis tank (Jencons Scientific Ltd, Beds.). Finally, electrode buffer (0.025M Tris, 0.192M glycine, 0.1%SDS, pH8.3) was added.

LPS samples or an equal volume of single strength PAGE sample buffer were then loaded into the wells of the stacking gel. The samples were run through the stacking gel at constant voltage of 60V and through the separating gel at 150V. After electrophoresis, LPS samples were examined by silver stain method (section 2.12.3) and the protein contaminants detected by colloidal gold total protein stain (section 2.12.5) after the gel was transferred to nitrocellulose (section 2.12.4).

Table 2.9 Preparation of separating and stacking PAGE gels

Reagent:	12% Separating gel	4% Stacking gel
Distilled Water	5.2ml	3.5ml
Separating buffer	17.5ml	------
Stacking buffer	------	5.0ml
Acrylamide solution (40%)	10.5ml	1.0ml
TEMED (Sigma)	50µl	20µl
Ammonium persulphate w/v (15mg/ml)	1.75ml	0.5ml

2.12.3 Silver stain of PAGE gel for LPS

LPS samples were visualized on PAGE gel according to the method proposed by Tsai and Frasch (1982) and modified by Hancock and Poxton (1988). PAGE gel was transferred into a tray containing fixative (25% propan-2-ol, 7% acetic acid) and left overnight at room temperature. The fixative was then discarded and the oxidiser (0.7% periodic acid in dilute fixative: 1.05g periodic acid, 4ml fixative and 150ml distilled water) was added for 15min at room temperature with slow agitation on a platform (used for all of the following steps). Oxidiser was discarded and gel was then washed in four changes of 200ml distilled water over 4h. Freshly prepared silver stain solution was prepared by adding 21ml 0.36% NaOH and 1.4ml 0.88 SG ammonia solution to 100ml distilled water, 4ml 19.4% silver nitrate solution was then added dropwise. Silver stain solution was added for 15min and then discarded. The gel was washed in four changes of 200ml distilled water over 40min. Samples were visualised using freshly prepared developer solution (0.005% citric acid in 0.019% formaldehyde solution) to achieve the required staining intensity. The developer was then discarded and the gel washed repeatedly in large volume of distilled water and finally scanned digitally.

2.12.4 Transferring gel to nitrocellulose

Components present in the gel after electrophoresis were transferred to a nitrocellulose membrane in a plastic cassette which was placed into electrophoresis tank. The electrode buffer was then poured into cover the whole of the cassette. A constant voltage of 40mA overnight at 4°C was used.

2.12.5 Colloidal gold total protein stain

For detecting protein contaminants bound to LPS samples, the method as described by Rohringer and Holden (1985) was used. The nitrocellulose membrane was transferred to an incubation vessel and washed three times, each for 20min, with 100ml of Tween Tris buffered saline (TTBS) (0.02M Tris/HCl, 0.5M NaCl pH 7.5, 0.025% Tween 20). The TTBS was then discarded and the membrane was washed three times, each for 2min, with 100ml of PFW. LPS protein bands were visualized after 50 ml of colloidal gold total protein stain (Bio-Rad) was added to the membrane. Once the desired staining intensity had been obtained the membrane was washed repeatedly in water, allowed to dry, digital scanned and recorded.

2.12.6 *Limulus* amoebocyte lysate assay (LAL assay)

The endotoxic activity of lyophilized preparation of different LPSs was determined by an endpoint method of LAL-assay using the Pyrochrome LAL kit (see section 2.3) which is based on the original work of Levin and Bang (1964). As described in the manufacturers instructions, Pyrochrome reagent was resuspended in 3.2ml of Pyrochrome buffer and kept in ice. To achieve a 1.0 EU/ml concentration, 2ml of LAL reagent water (LRW) was added to a vial containing 0.2ng of control endotoxin of *E.coli*. Applying doubling dilutions from 1.0 EU/ml (0.1ng/m) downwards, a 7 points standard curve was created. Samples of LPS preparations (10pg/ml and 100pg/ml) resuspended in LRW were then added at 50µl per well in a 96 well plate. PFW was included as a control.

A 50µl volume of reconstituted Pyrochrome reagent was then added to each sample or control and the plate was shaken gently, incubated at 37°C for 30 min. A 25µl volume of 50% acetic acid in PFW was then added to each well in order to stop the reaction. Absorbance values were read at 405nm on an Anthos 2001 automated plate reader.

2.13 Preparation of human monocytes for the bioassay

2.13.1 Media and reagents

Medium used to maintain human monocytes was prepared by adding 5 ml of human serum (HS:Sigma) to 50 ml of RPMI 1640 medium (RPMI/10%HS). White cell diluting fluid, WCDF was prepared by mixing 0.01% of Gentian Violet in 1% acetic acid. Crystal violet for TNF-α bioassay was prepared by mixing 0.5% Crystal Violet in 20% methanol in PFW and filtered through Whatman no.1 paper. Stocks of LPS samples were prepared at 1mg/ml by adding 5mg to 5ml of PFW then adding 5µl of triethlyamine (TEA) to completely dissolve the LPS. Human recombinant TNF-α for a standard curve control was purchased from NIBSC, (Hertfordshire, UK).

2.13.2 Separation of peripheral blood mononuclear cells (PBMC) from blood

Whole venous blood samples were obtained aseptically from healthy volunteers and placed in sterile heparinised tubes. One volume of blood sample was added to 3 volumes of sterile PBS. The mixture was then smoothly layered onto 10ml of pre-warmed Histopaque-1077 (Sigma) at 37°C. Red blood cells and granulocytes were separated from the PBMC following centrifugation at 300g for 30min at room temperature. The interface layer containing PBMC was carefully transferred by a sterile plastic pipette to a centrifuge tube and washed once with PBS and then RPMI 1640 medium at 300g for 7min. Finally PBMC was then resuspended in appropriate medium at the required concentration.

2.13.3 Counting of white blood cells

Pelleted PBMC were resuspended in 10ml of RPMI/10%HS. Of this suspension, 10µl was added to 90µl of WCDF. One chamber of an assembled haemocytometer (Hawksley, London) was then filled with a portion of the sample. Only monocytes cells which are characterised by an irregular nucleus and a prominent extended cytoplasm were counted for the purpose of monocytes plating experiment.

2.13.4 Separation of monocytes from PBMC

PBMC were resuspended at 2×10^5 monocytes per ml, plated at 100µl per well of a 96 well plate (Iwaki, Stone, UK) and incubated at 37°C for 1h in order to allow adhering of monocytes. Non-adherent cells were then removed with moderate shaking. Cells were then washed 3-4 times with sterile PBS and then resuspended in a medium as outlined in section 2.16.

2.14 Preparation of THP-1 cell line for bioassay

The human THP-1 cell line was used in parallel with human PBMC to measure the cytokine responses. After confluency was reached, THP-1 cells were resuspended at 5×10^5 cells/ml in RPMI/10%FCS. Untreated suspension was seeded at 100µl in each well of a 96-well tissue culture plate and cells were permitted to adhere by incubation at 37°C, 5% CO_2 overnight. For pre-treated suspension, 2ml of working concentration (=1µM) of Vitamin D3 (1,25-dihydroxyvitamin D3, Sigma) were add to every 20ml of the cell suspension (final concentration = 0.1µM) in order to differentiate THP-1 cells into the macrophage-like lineage. 100µl of this suspension was added to each well of 96-well tissue culture plate, incubated for 72h at 37°C and 5% CO_2. After incubation, medium was gently removed from both untreated and pre-treated cells, and directly replaced by 100µl of serial dilutions of LPS samples (see section 2.17)

2.15 Preparation of LPS dilutions

In a new 24-well culture plate, 10 fold × 7 times serial dilutions were created in RPMI/10%HS medium. Defrosted stock of LPS (10µl) at 1mg/ml were added to 990µl of the medium to achieve 10µg/ml, from which, 30µl were added to 270µl of the medium to achieve 1µg/ml (=1000ng/ml) as a starter concentration. The previous step was repeated across the plate to achieve the rest of six decreasing concentrations (1000ng, 100ng, 10ng, 1ng, 0.1ng and 0.01ng).

A cocktail of LPSs experiment in which a constant concentration of 200ng/ml from one type of LPS (i.e LPS1) mixed with serial dilutions of another type of LPS (i.e LPS2) were also done. Defrosted stock of LPS (10µl) at 1mg/ml were added to 990µl of the medium to achieve 10µg/ml then 20µl of this were added to 980µl of the medium to get the final concentration of 200ng/ml .

The serial dilution of LPS2 were achieved by adding 10µl of defrosted stock of LPS2 at 1mg/ml to 990µl of the medium to achieve 10µg/ml. From this, 50µl were added to 250µl of the medium to achieve 2µg/ml (=2000ng/ml) as a starter concentration. The previous step was repeated across the plate to achieve the rest of six decreasing concentrations (2000ng, 200ng, 20ng, 2ng, 0.2ng and 0.02ng).

When cocktail experiment was attempted 50µl of constant concentration of LPS1 were added to 50µl of the corresponding dilution of LPS2 so that all concentrations were decreased by half.

The constant concentration of LPS1 alone was also tried by adding 10µl of defrosted stock of LPS1 at 1mg/ml to 990µl of the medium to achieve 10µg/ml. From this 10µl were added to 990µl of the medium to achieve 100ng/ml. 100µl volume from this concentration was examined with each cocktail experiment plate.

2.16 Challenging of monocytes or THP-1 cells with LPS

A 100µl volume of the series of six LPS dilutions described in the previous section were added to the plated human monocytes or THP-1 cells prepared as in sections 2.13.4 and 2.14 respectively. Cell culture plates were then incubated at 37°C for 4h. Some wells were left with medium alone as a control. Finally, supernatants were collected and cytokine levels measured .

2.17 Cytokines detecting assays

2.17.1 L929 bioassay

L929 cells were used in a bioassay since they are sensitive to the cytotoxic effect of bioactive TNF-α. A method based on the work of Delahooke *et al.*(1995) was applied. Sub-confluent monolayers of L929 cells were trypsinized using the trypsin/EDTA method (section 2.5.1) and resuspended at 4×10^6 cells/ml in DMEM/5%FCS. Aliquots of 100µl of the cell suspension were then seeded in each well of 96-well tissue culture plate and cells were permitted to adhere for 3-4h of incubation.

Supernatants were then carefully aspirated from each well and replaced with 90µl (sample wells) or 100µl (standard curve wells) of DMEM/5%FCS/Act. The standard curve was created by removing 10µl of medium from well number one and replaced with 10µl of 100,000 IU/ml recombinant human TNF-α in PBS (NIBSC, Hertfordshire, UK) to make the concentration in the first wall of 10,000 IU/ml. A 3.2 fold serial dilutions arise from well number one was made along the standard curve across the plate by taking 45µl along and mixing into the 100µl already present. The final 45µl was removed from the last well in the row. For sample measurement, 10µl of fresh supernatant from challenged cells was transferred to the 90µl of DMEM/5%FCS/Act already present in each sample measurement well. Plates were then incubated at 37°C, 5% CO_2 overnight.

The next day, medium was discarded by inverting the plates. Viable cells were then stained with 50µl of L929 assay crystal violet which was added to each well and left for 3-4min, subsequently washed thoroughly under the tap, and briefly dried on tissue to remove the excess water. 50µl of L929 assay acetic acid (20% acetic acid in distilled water) was then added to each well to solubilise the stained cells. Optical density was measured with an automated microplate reader (Anthos 2001) at 540nm and all values were reported as the mean of triplicate measurements and compared to the standard curve.

2.17.2 Enzyme linked immunosorbent assay (ELISA)

Sandwich enzyme-linked immunosorbent assay of DuoSet system from R&D (Abingdon,UK) was used to detect the immuno-reactive TNF-α or IL-1β levels according to the manufacturer's instructions. A 100µl of working concentration (4µg/ml for both TNF-α and IL-1β) of capture antibody were added to each well of 96-well tissue culture plate, sealed and incubated overnight at room temperature. The plate was then aspirated, washed three times with wash buffer (0.05% Tween 20 in sterile PBS) at 400µl/well and inverted against clean paper towels to remove any remaining wash buffer. A 300µl of Blocking buffer (1% Bovine serum albumin, BSA (Sigma) in sterile PBS) was then applied for a minimum of 1h at room temperature and additional aspiration/wash step was then repeated.

Fresh sample supernatants (100µl per well) from challenged cells (see section 2.16) were transferred to the plate in addition to two internal standard curves which were included in every plate to measure the concentration of TNF-α or IL-1β in sample.

The internal standard curve for TNF-α ELISA assay was achieved by reconstituting 290ng/ml of ELISA recombinant human TNF-α with 0.5ml of filtered reagent diluent (1% bovine serum albumin, BSA (Sigma) in sterile PBS). From which, 10µl was added to 2900µl of filtered reagent diluent to achieve the working concentration of 1ng/ml.

The standard curve wells were filled by 600µl of filtered reagent diluent and 2-fold serial dilutions were achieved by transfer and well mix 600µl from the working concentration of recombinant human TNF-α along seven wells of this standard curve. The highest standard curve concentration of 1ng/ml was included followed by 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.25pg/ml and 15.625pg/ml.

The internal standard curve for IL-1β ELISA assay was achieved by reconstituting 110ng/ml of ELISA recombinant human IL-1β with 0.5ml of filtered reagent diluent. From which, 6µl was added to 2640µl of filtered reagent diluent to achieve the working concentration of 250pg/ml. The standard curve wells were filled by 600µl of filtered reagent diluent and 2-fold serial dilutions were achieved by transfer and well mix 600µl from the working concentration of recombinant human IL-1β along seven wells of this standard curve. The highest standard curve concentration of 250pg/ml was included followed by 125pg/ml, 62.5pg/ml, 31.25pg/ml, 15.625pg/ml, 7.8125pg/ml and 3.90625pg/ml.

The plate was then covered, incubated for 2h at room temperature and a third aspiration/wash step was then followed. A 100µl volume of working concentration of detection antibody (75ng/ml for TNF-α and 100ng/ml for IL-1β) was applied to each well of the plate, sealed and incubated for 2h at room temperature and a fourth aspiration/wash step was then followed. Avoiding direct light for the rest of the assay, 100µl of working conc (50µl in 10ml of reagent diluent) of Streptavidin-HRP were added to each well of the plate, incubated for 20min at room temperature and a final aspiration/wash step was then followed. 100µl of a complete substrate solution (1 tablet TMB (Sigma) dissolved in 10ml citrate phosphate buffer and 2µl of 30%H_2O_2) were then added to each well of the plate, incubated for 20min at room temperature and finally the colour reaction was stopped by adding 50µl of Stop solution (1M H_2SO_4) to each well, the plate was tapped to ensure thorough mixing. Absorbance optical density was measured with an automated microplate reader (Anthos 2001) at 450nm, all values were reported as the mean of triplicate measurements and compared to standard curve.

2.18 Toll like receptor assay

2.18.1 Plasmids

The following expression plasmids were a kind gift from Dr Clett Erridge (University of Strathclyde, Glasgow, UK):

1) pELAM stands for NF-κB dependent endothelial leukocyte adhesion molecule 1 promoter Luciferase, it is also called NF- κB dependent E-selectin promoter (Schindler and Baichwal, 1994; Chow *et al.*, 1999; Erridge *et al.*, 2007b).

2) The pRL plasmid is designed to express renilla luciferase driven by a cytomegalovirus promoter and was used as an internal control for efficiency of transfection.

3) The pCMV plasmid acts as a co-transfectant to maintain the quantity of DNA constant between transfections.

4) pCD14 expresses human cDNA for CD14

5) pTLR2 expresses human cDNA for TLR2

6) pTLR4 expresses human cDNA for both TLR4 and MD2

2.18.2 Transient transfection of HEK-293 cell line

To give 60-70% confluence at the day of transfection, HEK-293 cells were grown at an initial density of 8×10^4 cells per ml, plated at 100µl per well of 96-well plate and incubated at 37°C, 5% CO_2 the night prior to transfection. The following day, 168µl of DMEM/0% FCS were added to three sterile eppendorf tubes labelled TLR2, TLR4 and CD14. 6.3µl of GeneJuice transfection reagent (Novagen) were then added to the three tubes, briefly vortexed and allowed to sit for 5min at room temperature.

Plasmid constructs were then defrosted, added to the three tubes as outlined in Table 2.9, smoothly mixed and allowed to sit for 10min at room temperature. Carefully, 5.6µl from each tube were added to 24 wells of 96-well plated HEK-293 cells and incubated again at the same conditions for 48-72h.

Table 2.10 Components of plasmid constructs

Plasmid constructs	Contents of tubes		
	TLR2	TLR4	CD14
pELAM (10ng)	0.9µl	0.9µl	0.9µl
pCD14 (30ng)	1.6µl	1.6µl	1.6µl
pCMV	1.8µl	------	2.7µl
pRL	2.4µl	2.4µl	2.4µl
pTLR2 (10ng)	3.3µl	------	------
pTLR4 (30ng)	------	5.4µl	------

2.18.3 Stimulation of transfected HEK-293 cell

LPS and heat killed bacteria (HKB) samples were made up in DMEM/1% FCS. The medium was then removed from the transfected cell culture plates (previous section) and quickly replaced by 120µl per well of either LPS or HKB samples. One row was left with medium alone, another with *E. coli* LPS as a TLR4 positive control and a third lane with *P. gingivalis* LPS as TLR2 positive control. The plate was then returned to the incubator at 37°C, 5% CO_2 overnight.

2.18.4 Luciferase assay

Two Luciferase assays were applied, firefly luciferase which is a 61kDa protein isolated from beetles (*Photinus pyralis*) and Renilla luciferase which is a 36kDa protein from sea pansy (*Renilla reniformis*) that was used to normalize and control the transfection process in the firefly luciferase assay (Matijasevic *et al.*, 2001; Nieuwenhuijsen *et al.*, 2003).

The following day after stimulation of transfected HEK-293 cell, 10ml per plate of lysis buffer were defrosted. This lysis buffer was prepared by adding 168 ml distilled water to 25 mM Tris (605 mg), 8 mM $MgCl_2.6H_2O$ (325 mg), 1 mM DTT (30.8 mg), 1% Triton X-100 (2 ml) and 30ml glycerol. The pH was then adjusted to 7.8 with phosphoric acid.

A sufficient amount of luminescent reagent, either for firefly luciferase or Renilla luciferase was also defrosted. Firefly luciferase luminescent reagent buffer was prepared by adding 1 mM ATP (36.5 mg), 0.25 mM Lucferin (5 mg) (Sigma), 1% BSA (662 mg) to 66.2ml of lysis buffer. Renilla luciferase luminescent reagent buffer was prepared by dissolving Coelenterazine to 1mg/ml in absolute EtOH then aliquoted into foil wrapped eppendorfs then stored desiccated at –80°C. It was then diluted 1:500 in room temperature PBS just before use.

Plated cells were washed with 120µl per well of PBS, then lysed by adding 100µl per well of lysis buffer. Using a multichannel pipette, wells were then scratched several times in a circular motion, pipetted up and down several times to ensure all cells were lysed. A 40µl sample from each well was then transferred to a white luminometer plate and either one of the previous luminescent reagent buffers was set up to inject 40µl per well. Finally, the plate was placed in a BMG Lumistar Galaxy and read according to manufacturer's instructions

The fold expression was presented in the graph and calculated as counts from pELAM expression divided by counts from pRL which gives raw promoter expression (RPE). Fold induction is the RPE for treated cells divided by the RPE value for cells cultured in medium alone.

2.19 Statistical analysis

The results depicted in section 3.2 to section 3.7 are expressed as means +/- SEM of at least three independent experiments and analysed by student t-test. P values of

$p< 0.05$ were considered signiicant.

CHAPTER 3: RESULTS

3.1 Lipopolysaccharides analysis

LPS preparations were extracted by 4 different extraction methods and all of them were further repurified by the method described in (section 2.11).

All of the 4 methods have been successfully applied to *E. coli*, *B. fragilis*, *Ps. aeruginosa*. LPS extracted by PCP method from *B. fragilis* showed the least yield and the hardest to solubilise. *R. sphaeroides* LPS has been extracted by two methods, PCP and PW.. As shown in Table 3.1, yields of LPSs extracted by these methods and the percentage of the extracted LPS from the dry weight of the bacterial cell varied between methods. The highest LPS yield seems to be that extracted by BW method from *E. coli*, *B. fragilis* and *Ps. aeruginosa*. The lowest LPS yield seems to be that extracted by TM/TMP method from *E. coli*. The next lowest LPS yield seems to be that extracted by PCP method from *B. fragilis*.

The structural morphology of the investigated LPS preparations were analysed by non-SDS PAGE:

3.1.1 Electrophoretic analysis of *E. coli* 018K⁻ LPS preparations

Three different methods were used to extract LPS form *E. coli*: TM/TMP, AP and BWP methods (Fig 3.1A, B). Four extracted LPS preparations were further repurified to remove any possible protein contaminants (Fig 3.1C).

The LPS preparations used in (Fig 3.1A) were submitted to non-SDS PAGE in which the LPS components travel in the gel according to their molecular weights, and LPSs were visualized by silver staining. An overall view of the *E.coli* LPS profiles demonstrated in Fig 3.1A, which represent the unpurified LPS preparations (EC1u, EC2u, EC3u and EC4u), reveals some differences between these four chemotypes which are resultant from different extraction methods as mentioned above. EC1u, EC2u, EC3u, which were extracted by TMP, TM and AP respectively, show an almost equal intensity in the low-molecular mass of Lipid A regions seen in the base line of the gel (labeled R), EC3u shows the most intense one. On the other hand, EC4u, which was extracted by BWP, seems to be faint in that low-molecular mass region compared with the other three profiles. Nevertheless, all of this fast-migrating

material of the 4 LPS profiles are discernible as a doublet of bands. Similarly, common antigen (CA) band, which is present behind the main front band, has almost the same intensity in EC1u, EC2u and EC3u but it is very faint in EC4u.

Table 3.1 Yields of different LPSs obtained by different extraction methods

Strain	E. coli O18K⁻			Ps. aeruginosa Pa-O1			B. fragilis NCTC 9343		
	cell dw	LPS dw	%	cell dw	LPS dw	%	cell dw	LPS dw	%
AP	5.4g	64.5mg	1.2%	3.3g	12.9mg	0.4%	-	-	-
TM	2g	1.1mg	0.06%	2g	36mg	1.8%	2g	15.1mg	0.76%
TMP	2g	2.9mg	0.15%	2g	19.1mg	0.96%	2g	27.3mg	1.37%
BW	180mg	18.1mg	10.1%	200mg	20.3mg	10.15%	250mg	12.8mg	5.12%
PCP	-	-	-	-	-	-	3.6g	8.1mg	0.23%

(AP) = aqueous phenol method, (TM) = Triton/magnesium chloride method, (TMP) = Triton/magnesium chloride plus proteinase K treatment method, (BW) = Boiling water/ Proteinase K treatment method, (PCP) = Phenol/chloroform/petroleum spirit method, (cell dw) = dry weight of the bacterial cell before LPS extraction, (LPS dw) = dry weight of the specific LPS, (%) = percentage of the extracted LPS from the bacterial cell dry weight.

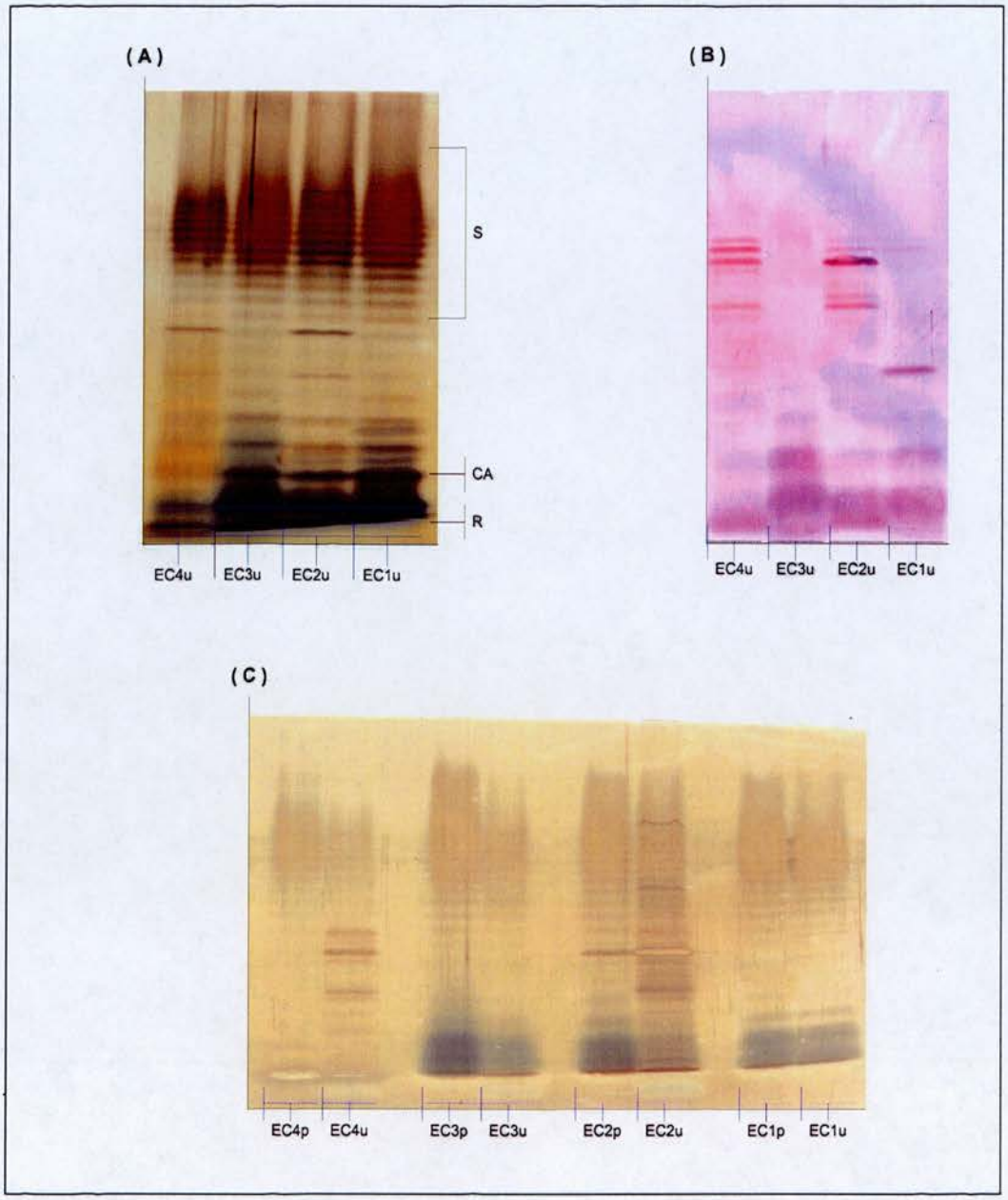

Figure 3.1 Analysis of E. coli 018K- LPS preparations by non-SDS PAGE and colloidal gold total protein

(A) = non-SDS PAGE for unpurified LPSs, (B) = colloidal gold stain for unpurified LPSs, (C) = non-SDS PAGE for unpurified and purified LPSs, (EC1u) = unpurified *E. coli* LPS extracted by TMP, (EC1p) = purified EC1u LPS, (EC2u) = unpurified *E. coli* LPS extracted by TM, (EC2p) = purified EC2u LPS, (EC3u) = unpurified *E. coli* LPS extracted by AP, (EC3p) = purified EC3u LPS, (EC4u) = unpurified *E. coli* LPS extracted by BWP, (EC4p) = purified EC4u LPS, (R) = rough LPS at gel front, (CA) = common antigen, (S) = ladder pattern of smooth LPS.

All of these four chemotypes yielded the classical ladder-like O-antigenic domain profiles of smooth LPS. Moreover, the migration pattern of these high-molecular weight bands are almost identical. In this stage of LPS analysis, EC1u and EC3u demonstrate the much similar patterns to the classical LPS.

The LPS preparations were submitted to non-SDS PAGE transferred to nitrocellulose membrane and subjected to colloidal gold staining to detect any protein contaminants (Fig 3.1B). For the rough type regions, since EC4u has the most faint rough region in Fig 3.1A, it obviously shows the most rough domain free from protein contaminants followed by EC1u and EC2u as they both have same protein materials in the rough type regions. Parallel to the level of intensity of the rough type region shown in Fig 3.1A, EC4u also shows the most rough type region that contains most attached protein materials to both rough type and common antigen regions as well. The clearest CA region from protein is EC4u since it is the most faint one in PAGE gel followed by EC2u then EC1u (Fig 3.1B). However, the main difference in the content of protein material is demonstrated in the high-molecular weight bands. EC3u seems to have the cleanest smooth type that is almost free from protein followed by EC1u which is extracted by TMP. On the other hand EC2u has higher protein contaminants since it was extracted by TM only. Although EC4u LPS was extracted by BWP which is include proteinase K treatment, it contains the most protein materials in the smooth type regions.

The LPS preparations were submitted to non-SDS PAGE, and LPSs were visualized by silver staining (Fig 3.1C). An overall view of the *E.coli* LPS profiles demonstrated the unpurified and purified LPS preparations (EC1u, EC1p, EC2u, EC2p, EC3u, EC3p, EC4u, and EC4p), directly reveals a noticeable drop in protein contents after applying the repurification method to eliminate the protein contaminants except between LPS profiles extracted by TMP and AP respectively (EC1u, EC1p, EC3u, and EC3p) which are nearly identical in the gel before and after repurification. EC4p appears to be the LPS that contains the least protein followed by EC1p. Although EC2p demonstrates a more protein free profile than EC2u, it still shows a clear band just under the ladder pattern region that is probably protein in nature.

3.1.2 Electrophoretic analysis of *B. fragilis* LPS preparations

Four different methods were used to extract LPS form *B. fragilis*: TM/TMP, PCP, BWP and AP (Fig 3.2A, B). Five extracted LPS preparations were further repurified to remove any possible protein contaminants (Fig 3.2C).

The LPS preparations were subjected to non-SDS PAGE and the LPS visualized by silver staining (Fig 3.2A). LPS profiles which represent the unpurified LPS preparations (BF1u, BF2u, BF3u and BF4u), reveals also some differences between these four chemotypes which are a result of the different extraction methods as mentioned above. BF1u, BF2u and BF3u which were extracted by TM, TMP and PCP respectively, show an almost equal intensity in the low-molecular mass of Lipid A regions seen in the base line of gel (labeled R) with the most intense pattern in BF2u. On the other hand, BF4u, which was extracted by BWP, seems to be faint in that low-molecular mass region compared with the other three profiles. The high molecular weight bands were more defined in BF1u and BF2u. While BF3u show major diffuse material located just behind the fast-migrating band. To a lesser extent, the appearance of BF4u profile is similar to that of BF3u in this region.

The LPS preparations used in Fig 3.2B were submitted to non-SDS PAGE transferred to nitrocellulose membrane and proteins present visualized by colloidal gold staining. BF1u and BF4u showed a rough region almost clear from protein material. The rough type area of BF2u appeared to have very little protein associated with it while BF3u shows a little protein material in its rough region. The main differences were concentrated in the higher molecular weight bands, in which BF2u shows the clearest profile among not only *B. fragilis* LPS profiles but also among all examined LPS profiles before the repurification method was applied. BF1u and BF4u show some protein contaminated bands in their high-molecular weight regions. But the worst scene is shown in BF3u profile which contains high level of protein contaminants diffused along its high-molecular weight region.

A comparison between unpurified LPS preparations (BF1u, BF2u, BF3u, BF4u and BF5u) and repurified LPS preparations (BF1p, BF2p, BF3p, BF4p and BF5p) is seen in Fig 3.2C. It considerably demonstrates clear profiles of repurified LPS preparations after the repurification procedure was applied.

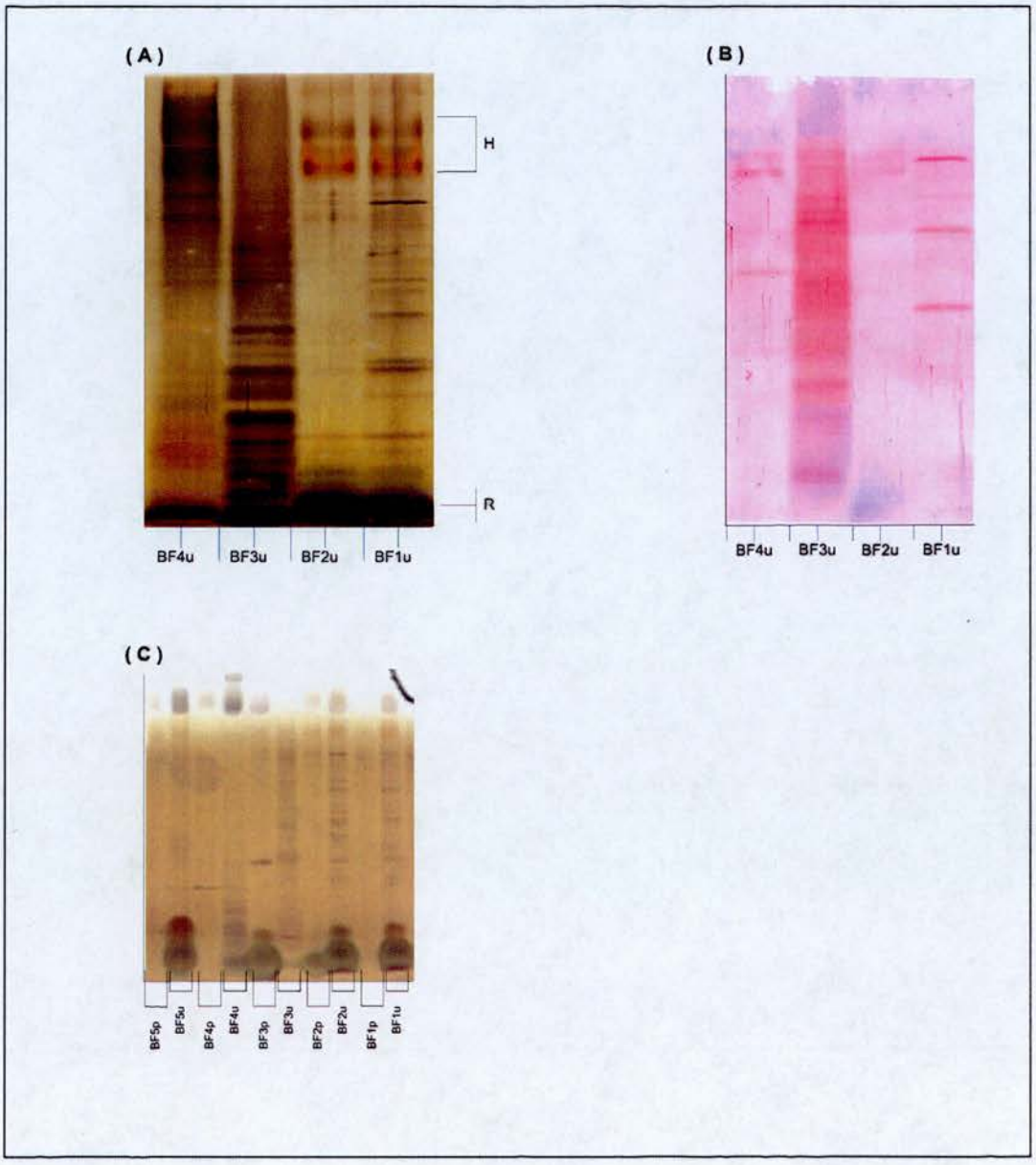

Figure 3.2 Analysis of *B. fragilis* NCTC 9343 LPS preparations by non-SDS PAGE and colloidal gold total protein

(A) = non-SDS PAGE for unpurified LPSs, (B) = colloidal gold stain for unpurified LPSs, (C) = non-SDS PAGE for unpurified and purified LPSs, (BF1u) = unpurified *B. fragilis* LPS extracted by TM, (BF1p) = purified BF1u LPS, (BF2u) = unpurified *B. fragilis* LPS extracted by TMP, (BF2p) = purified BF2u LPS, (BF3u) = unpurified *B. fragilis* LPS extracted by PCP, (BF3p) = purified BF3u LPS, (BF4u) = unpurified *B. fragilis* LPS extracted by BWP, (BF4p) = purified BF4u LPS, (BF5u) = unpurified *B. fragilis* LPS extracted by AP, (BF5p) = purified BF5u LPS, (R) = rough LPS at gel front, (H) =high molecular weight region.

3.1.3 Electrophoretic analysis of *Ps. aeruginosa* PA-01 L and *R. sphaeroides* LPS preparations

Three different methods were used to extract LPS form *Ps. aeruginosa*: TM/TMP, AP and BWP methods (Fig 3.3A, B). AP and PCP methods were used to extract LPS from *R. sphaeroides* and Fig 3.3C shows *R. sphaeroides* LPS which was extracted by AP method. The *Ps. aeruginosa* LPS preparations were subjected to non-SDS PAGE and the LPSs were visualized by silver staining (Fig 3.3A). An overall view of the *Ps. aeruginosa* LPS profiles demonstrated in Fig 3.3A, which represent the unpurified LPS preparations (PA1u, PA2u, PA3u and PA4u), reveals some differences between these four chemotypes which are a result of the different extraction methods used. PA1u, PA2u and PA3u which were extracted by TM, TMP and AP respectively, show an almost equal intensity in the low-molecular mass Lipid A regions seen in the base line the of gel with most intense pattern in PA2u. On the other hand, PA4u, which was extracted by BWP, seems to have only a faint profile in that low-molecular mass region compared with the other three profiles. The ladder pattern of smooth type region is seen in the four LPS profiles with a classical and distinguished appearance in PA1u and PA2u. PA3u and PA4u exhibit a closely spaced smooth type regions.

The LPS preparations used in (Fig 3.3B) were submitted to non-SDS PAGE transferred to nitrocellulose membrane and proteins present visualized by colloidal gold staining. There are very little differences between PA1u, PA2u and PA3u in the protein contaminants in their rough regions. They also have a protein contaminants in their common antigen regions. PA4u shows the least protein material not only in both rough and common antigen regions, but also in its smooth type regions. PA3u has the most contamination of the smooth type region with protein material whereas the proteinase K treatment does not make a noticeable difference between PA1u and PA2u in their high-molecular weight regions since the later was subjected to such treatment.

R. sphaeroides LPS was a difficult LPS to extract and many extraction methods were used. These methods included PCP with applying both further procedures of ether/acetone and water and TM procedures.

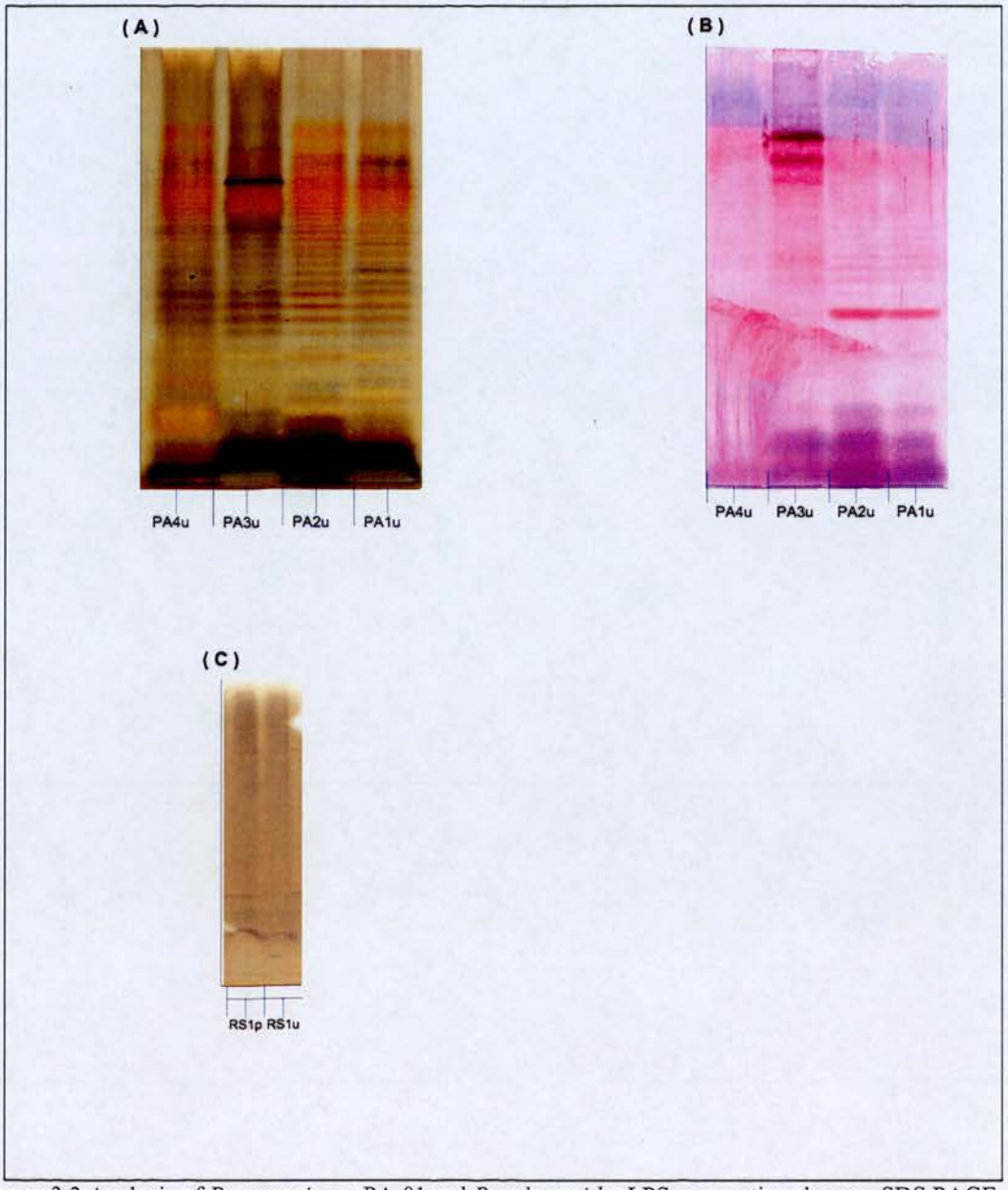

Figure 3.3 Analysis of *Ps. aeruginosa* PA-01 and *R. sphaeroides* LPS preparations by non-SDS PAGE and colloidal gold total protein

(A) = non-SDS PAGE for unpurified *Ps. aeruginosa* LPSs, (B) = colloidal gold stain for unpurified *Ps. aeruginosa* LPSs, (C) = non-SDS PAGE for unpurified and purified *R. sphaeroides* LPSs, (PA1u) = unpurified *Ps. aeruginosa* LPS extracted by TM, (PA1p) = purified PA1u LPS, (PA2u) = unpurified *Ps. aeruginosa* LPS extracted by TMP, (PA2p) = purified PA2u LPS, (PA3u) = unpurified *Ps. aeruginosa* LPS extracted by AP, (PA3p) = purified PA3u LPS, (PA4u) = unpurified *Ps. aeruginosa* LPS extracted by BWP, (PA4p) = purified PA4u LPS, (RS1u) = unpurified *R. sphaeroides* LPS extracted by AP, (RS1p) = purified RS1u LPS.

The only success of extracting this kind of LPS was when applying the AP procedure after a full depigmentation process (Fig 3.3C). However the author was given three previous small LPS stocks of this species most kindly extracted by Mr Robert Brown and Mr Fraser Pike. Fig 3.3C shows that there are not many differences between RS1u and RS1p and in addition they both exhibit a faint appearance.

3.2 Activity of LPS preparations in LAL assay

The endotoxic activity of all crude LPS preparations was measured by an endpoint method of LAL assay. All of *E. coli* LPS preparations reveal different levels of LAL activity as shown in Fig 3.4A either at 0.1ng/ml or 0.01ng/ml concentrations. EC3u which was extracted by AP method reveals the most active preparations at 0.1ng/ml that was significantly higher than all other four *E. coli* LPS preparations ($p< 0.05$) at 0.1ng/ml concentration. Nevertheless, EC3u was not statistically significant at 0.01ng/ml ($p< 0.05$). While EC1u which was extracted by TMP shows the least LAL activity. Also obvious is the huge differences in the LAL activity between 0.1ng/ml and 0.01ng/ml concentrations for all *E. coli* LPS preparations.

An almost identical scenario is repeated by *Ps. aeruginosa* LPS preparations which demonstrate different levels of LAL activity as shown in Fig 3.4A either at 0.1ng/ml or 0.01ng/ml concentrations. However, there is no much difference between PA1u and PA2u in their LAL activities. PA4u which was extracted by BWP method reveals the most active preparations among all *Ps. aeruginosa* LPS preparations at 0.1ng/ml concentrations but it was significantly higher when compared to PA3u only ($p< 0.05$). Again a large differences in the LAL activity between 0.1ng/ml and 0.01ng/ml concentrations for all *Ps. aeruginosa* LPS preparations is observed. EC1u, EC2u, EC3u, EC4u and PA4u showed a significant differences between 0.1ng/ml and 0.01ng/ml ($p< 0.05$).

B. fragilis crude and purified LPSs were tested again as shown in Fig 3.4B. It is obvious from Fig 3.4A that the LAL activity of most *B. fragilis* LPSs is much lower than the activity of other tested LPSs. However, BF5u is approximately fivefold more active than other *B. fragilis* LPSs at the concentration of 0.1ng/ml of LPS (Fig 3.4B). It also showed a significant statistical difference among all other *B. fragilis*

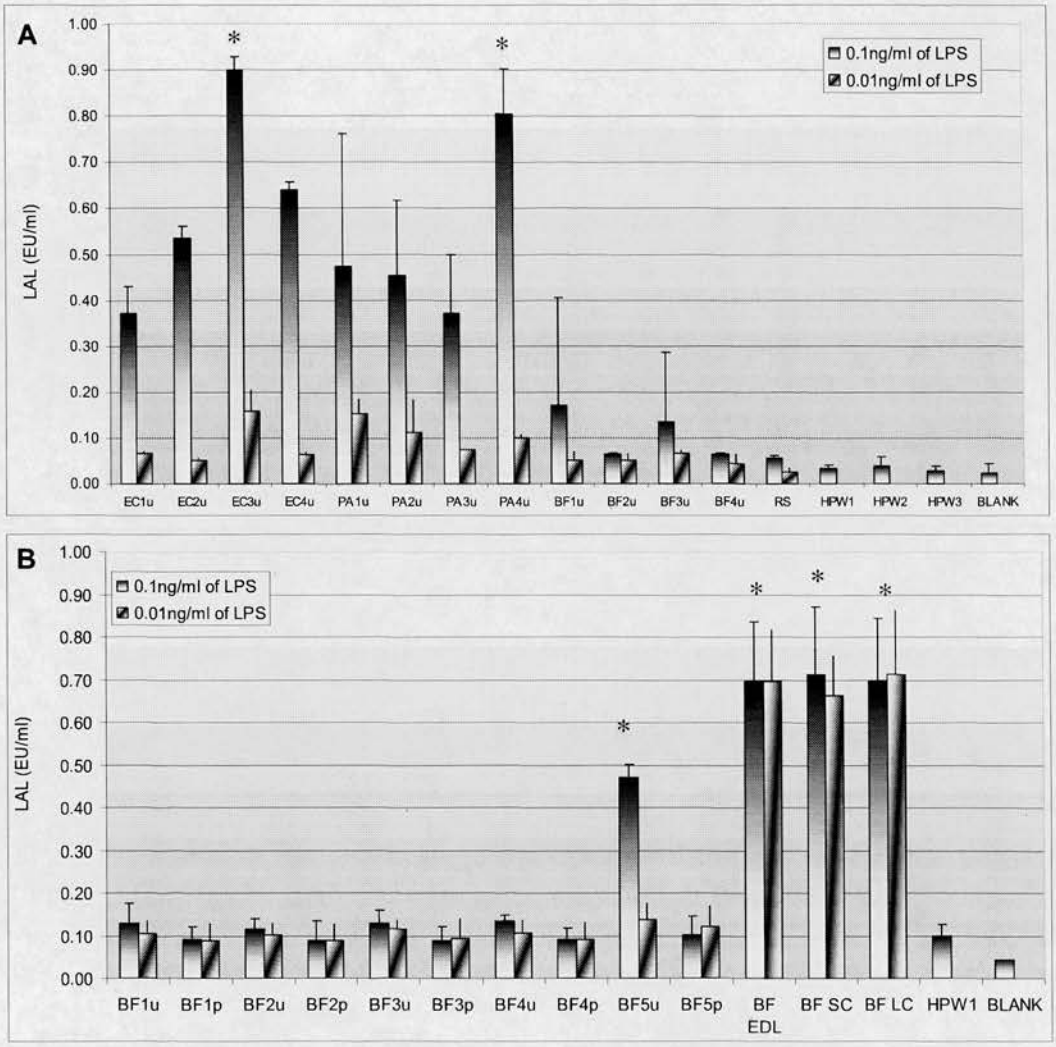

Figure 3.4 The LAL activity of different LPS preparations

(A) = LAL activity of unpurified LPSs, (B) = LAL activity of unpurified and purified *B. fragilis* LPSs, (BF1u) = unpurified *B. fragilis* LPS extracted by TM, (BF1p) = purified BF1u LPS, (BF2u) = unpurified *B. fragilis* LPS extracted by TMP, (BF2p) = purified BF2u LPS, (BF3u) = unpurified *B. fragilis* LPS extracted by PCP, (BF3p) = purified BF3u LPS, (BF4u) = unpurified *B. fragilis* LPS extracted by BWP, (BF4p) = purified BF4u LPS, (BF5u) = unpurified *B. fragilis* LPS extracted by AP, (BF5p) = purified BF5u LPS, (BF EDL) = Heat killed bacteria of *B. fragilis* of electronic dense layer population, (BF SC) = Heat killed bacteria of *B. fragilis* of small capsule population, (BF LC) = Heat killed bacteria of *B. fragilis* of large capsule population, (HPW1) = Injection pyrogen free water, (HPW2) = Highly purified water 1, (HPW3) = Highly purified water 2, (BLANK) = Empty wells, Results represent the means +/- SEM for at least two experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

crude and purified LPSs ($p< 0.05$) at 0.1ng/ml but not at 0.01ng/ml. Although BF1u and BF3u show a considerable higher activity in the data depicted in Fig 3.4A at the same concentration, these results probably reflect inconsistence readings from three experiments (0.06-0.066-0.39) and (0.07-0.052-0.28) for BF1u and BF3u respectively. There are no much differences between the activity of all *B. fragilis* LPSs at the concentration of 0.01ng/ml of LPS.

It is also reasonable that heat killed bacteria of *B. fragilis* from three different populations of different capsular polysaccharide all significantly showed high LAL activity compared to other LPS samples except BF5u ($p< 0.05$).

3.3 Different LPS preparations of the same bacterial species stimulated TNF-α production by human PBMC

3.3.1 Different LPSs of *E. coli* stimulated TNF-α production measured by L929 cell line

E. coli LPS preparations were picked to measure their ability to produce TNF-α from human PBMC isolated from five healthy volunteers and then measured by L929 cells which are sensitive to the cytotoxic effect of this specific cytokine (Fig 3.5). General view of these TNF-α responses show that there are reasonable differences at two higher concentrations (10μg/ml and 1μg/ml) among all these *E. coli* LPSs.

EC1u which was extracted by TMP reveals the highest ability to induce TNF-α production especially at 10μg/ml and 1μg/ml concentrations followed by EC3u which was extracted by AP. At 10μg/ml EC1u and EC3u were significantly higher than both EC2u and EC4u ($p< 0.05$). At 1μg/ml EC1u was significantly higher than all other *E. coli* LPS preparations whereas EC3u was significantly higher than EC4u only ($p< 0.05$). Furthermore, EC2u reveals low TNF-α production comparing with other *E. coli* LPSs and especially EC1u. EC1u and EC2u were extracted with the same method with the only difference being proteinase K treatment applied to the former.

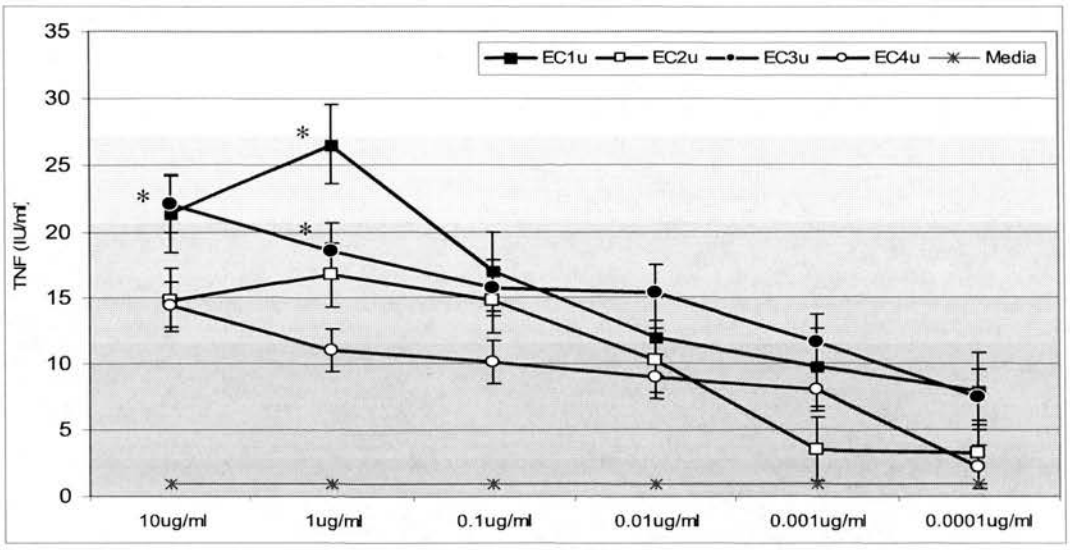

Figure 3.5 TNFα production (IU/ml) after 4 h by human PBMC after stimulation with different concentrations from different *E. coli* LPSs and measuring by L929

(EC1u) = unpurified *E. coli* LPS extracted by TMP, (EC2u) = unpurified *E. coli* LPS extracted by TM, (EC3u) = unpurified *E. coli* LPS extracted by AP, (EC4u) = unpurified *E. coli* LPS extracted by BWP. Results represent the means +/- SEM for at least three experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

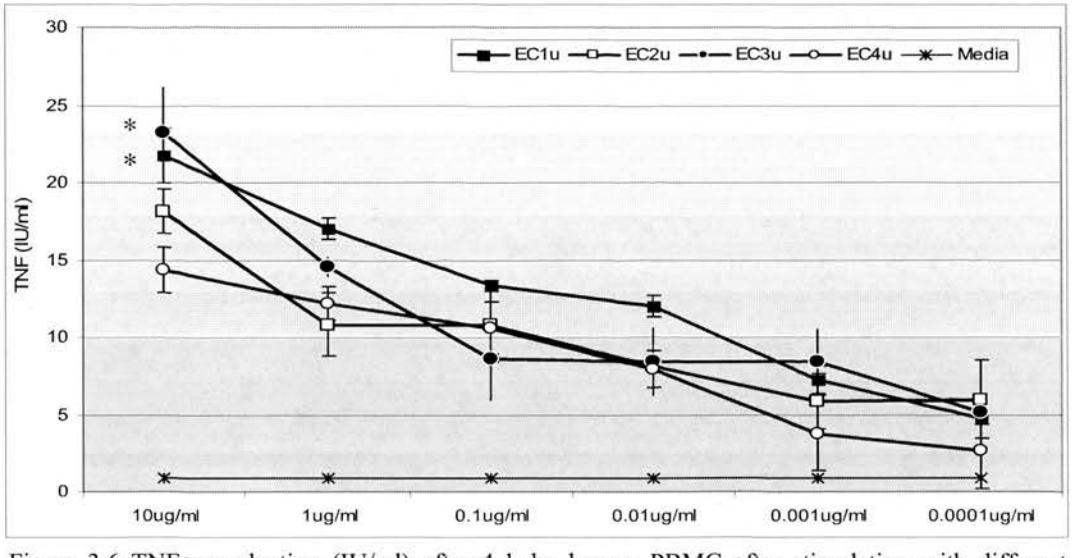

Figure 3.6 TNFα production (IU/ml) after 4 h by human PBMC after stimulation with different concentrations from different *E. coli* LPSs and measuring by ELISA

(EC1u) = unpurified *E. coli* LPS extracted by TMP, (EC2u) = unpurified *E. coli* LPS extracted by TM, (EC3u) = unpurified *E. coli* LPS extracted by AP, (EC4u) = unpurified *E. coli* LPS extracted by BWP. Results represent the means +/- SEM for at least three experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

3.3.2 Different LPSs of *E. coli* stimulated TNF-α production measured by ELISA

The same approach was used to measure the ability of *E. coli* LPS preparations to produce TNF-α via human PBMC but then measured by ELISA assay (Fig 3.6). Again EC1u and EC3u stimulated the most TNF-α production at the higher concentration of 10μg/ml and 1μg/ml. At 10μg/ml EC1u and EC3u were again significantly higher than both EC2u and EC4u ($p< 0.05$). But both of EC1u and EC3u were not significantly higher than EC2u and EC4u at 1μg/ml ($p< 0.05$).

There was a marked difference between the levels of TNF-α production of EC1u and EC2u despite the fact they are both extracted with the same method TM/TMP apart from proteinase K treatment in the latter. Once again EC4u exhibited the least amount of TNF-α production at the higher concentration of 10μg/ml and 1μg/ml (Fig 3.6). Both Fig 3.5 and Fig 3.6 demonstrate that TMP and AP methods produced LPSs that induce high TNF-α response compared to that of TM and BWP.

3.3.3 Different LPSs of *B. fragilis* stimulated TNF-α production measured by ELISA

B. fragilis LPS preparations were also chosen to compare their ability to produce TNF-α from human PBMC and then measured by ELISA (Fig 3.7). BF4u which was extracted by the mild method of BW showed the most significant TNF-α production at 10μg/ml LPS concentration compared to BF2u and BF3u but not BF1u. BF1u in turn showed a significant higher TNF-α production compared to BF3u but not BF2u ($p< 0.05$). At 1μg/ml, BF4u showed a significant higher TNF-α production compared to other three *B. fragilis* LPS preparations. Moreover, TNF-α production in response to BF1u remained significantly higher at lower concentrations 0.1μg/ml and 0.01μg/ml compared to BF3u and BF4u; and BF3u respectively. While BF3u which was extracted by the PCP method produced the least amount of that cytokine at the same concentration. Moreover, there was little difference between all of these *B. fragilis* LPSs at the concentration of 1μg/ml. Again BF3u which was extracted by PCP method demonstrated the least ability to produce such a TNF-α response at the lower concentrations of 0.1μg/ml, 0.01μg/ml, 0.001μg/ml, 0.0001μg/ml (Fig 3.7).

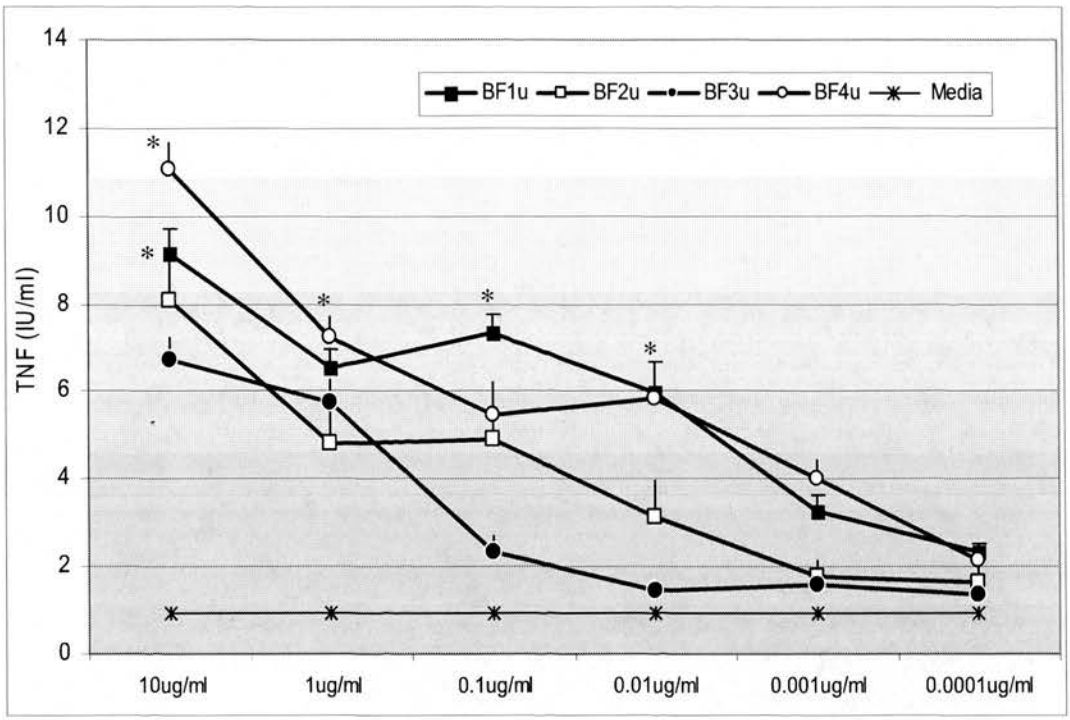

Figure 3.7 TNFα production (IU/ml) after 4 h by human PBMC after stimulation with different concentrations from different *B. fragilis* LPSs and measuring by ELISA

(BF1u) = unpurified *B. fragilis* LPS extracted by TM, (BF2u) = unpurified *B. fragilis* LPS extracted by TMP, (BF3u) = unpurified *B. fragilis* LPS extracted by PCP, (BF4u) = unpurified *B. fragilis* LPS extracted by BWP. Results represent the means +/- SEM for at least three experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

3.4 Interactions between different LPSs from various bacterial species in stimulation TNF-α production by human PBMC

3.4.1 Interactions between *E. coli* and *B. fragilis* LPSs

Using the ELISA assay to measure the TNF-α production after challenging human PBMC cells, with mixture of *E. coli* LPS preparations extracted by different methods with their equivalent *B. fragilis* LPS preparations (Fig 3.8, Fig 3.9, Fig 3.10, Fig 3.11).

In parts A of these figures, comparisons between various concentrations of *B. fragilis* LPS alone and various concentrations of *B. fragilis* LPS plus a constant concentration of *E. coli* LPS (100ng/ml) are displayed. The effect of a maximum *E. coli* concentration (100ng/ml) alone is also included. While parts B demonstrated the opposite approach in which comparisons were made between various concentrations of *E. coli* LPS alone and various concentrations of *E. coli* LPS plus a constant concentration of *B. fragilis* LPS (100ng/ml). The effect of *B. fragilis* LPS at concentration of 100ng/ml is also included. The third approach is depicted in parts C in which comparisons were made between concentrations of *E. coli* LPS alone, concentrations of *B. fragilis* LPS alone and equal concentrations of *E. coli* and *B. fragilis* LPSs included together the same time. An overall view of parts A in these figures gives the initial impression that *B. fragilis* LPS preparations exert a lowering and masking effect on TNF-α stimulation by the different *E. coli* LPS preparations especially at the higher concentrations of 1000ng/ml and 100ng/ml. In parts B, the ability of constant concentration (100ng/ml) of different *B. fragilis* LPS preparations to lower that of *E. coli* LPS preparations was clearly demonstrated with EC1u/BF1u at the lower concentrations of EC1u LPS (10ng/ml, 1ng/ml, 0.1ng/ml). This *B. fragilis* LPSs lowering effect was started at much lower concentration of 1ng/ml with EC3u/BF3u. On the other hand, it was started at higher concentration of 100ng/ml with EC4u/BF4u and EC2u/BF2u. However, in parts C, no uniformity of such lowering effect of *B. fragilis* LPSs could be deduced.

In Fig 3.8A, the lowering effect was significant when BF1u+constEC1u was compared to maxEC1u at 1000ng, 10ng, 1ng and 0.1ng ($p<0.05$). In Fig 3.8B,

EC1u+constBF1u was significantly higher than maxBF1u at only the first two higher concentrations whereas it was significantly lower than EC1u at lower concentrations of 10, 1 and 0.1ngs ($p<0.05$). In Fig 3.11C, EC1u/BF1u was significantly higher than BF1u at all concentrations ($p<0.05$).

Fig 3.9A showed more significant lowering effect than it was shown in Fig 3.8A. In Fig 3.9B, EC2u+constBF2u was significantly higher than maxBF2u at the first four higher concentrations while it was significantly lower than EC2u at only 100ng and 10ng ($p<0.05$). In Fig 3.11C, EC2u/BF2u was significantly higher than BF2u at all concentrations while it was significantly lower than EC2u at only 100 and 10ngs ($p<0.05$).

Fig 3.10A shows the least significant lowering effect of its type. In fact, BF3u+constEC3u was significantly lower than maxEC3u at only 1000ng and 100ng ($p<0.05$). In Fig 3.10B, EC3u+constBF3u was significantly higher than maxBF3u at the first three higher concentrations while there was no significant differences between EC3u+constBF3u and EC3u at any concentration. In Fig 3.10C, EC3u/BF3u was significantly higher than BF3u at 1000, 100, 1 and 0.01ngs ($p<0.05$), while there was no significant differences between EC3u/BF3u and EC3u at any concentration.

In Fig 3.11A, the lowering effect was so representative in such dose-dependent style with EC4u/BF4u which were both extracted by BW method. In fact BF4u+constEC4u was statistically significant from maxEC4u at 1000ng, 100ng, 1ng and 0.1ng. In Fig 3.11B, EC4u+constBF4u was significantly higher than maxBF4u at all concentrations while it was significantly lower than EC4u at only 1ng and 0.1ng. In Fig 3.11C, EC4u/BF4u was significantly higher than BF4u at all concentrations while it was significantly lower than EC4u at only 1000ng ($p<0.05$).

3.4.2 Interactions between different LPSs and *R. sphaeroides* LPS preparations

R. sphaeroides LPS was difficult to extract and RS1u represent the LPS preparation of this bacteria which was fully depigmented and then extracted by AP method. The effect of interactions between RS1u and LPSs from different bacterial species in production of TNF-α is demonstrated in Fig 3.12. As shown in Fig 3.12A, RS1 LPS

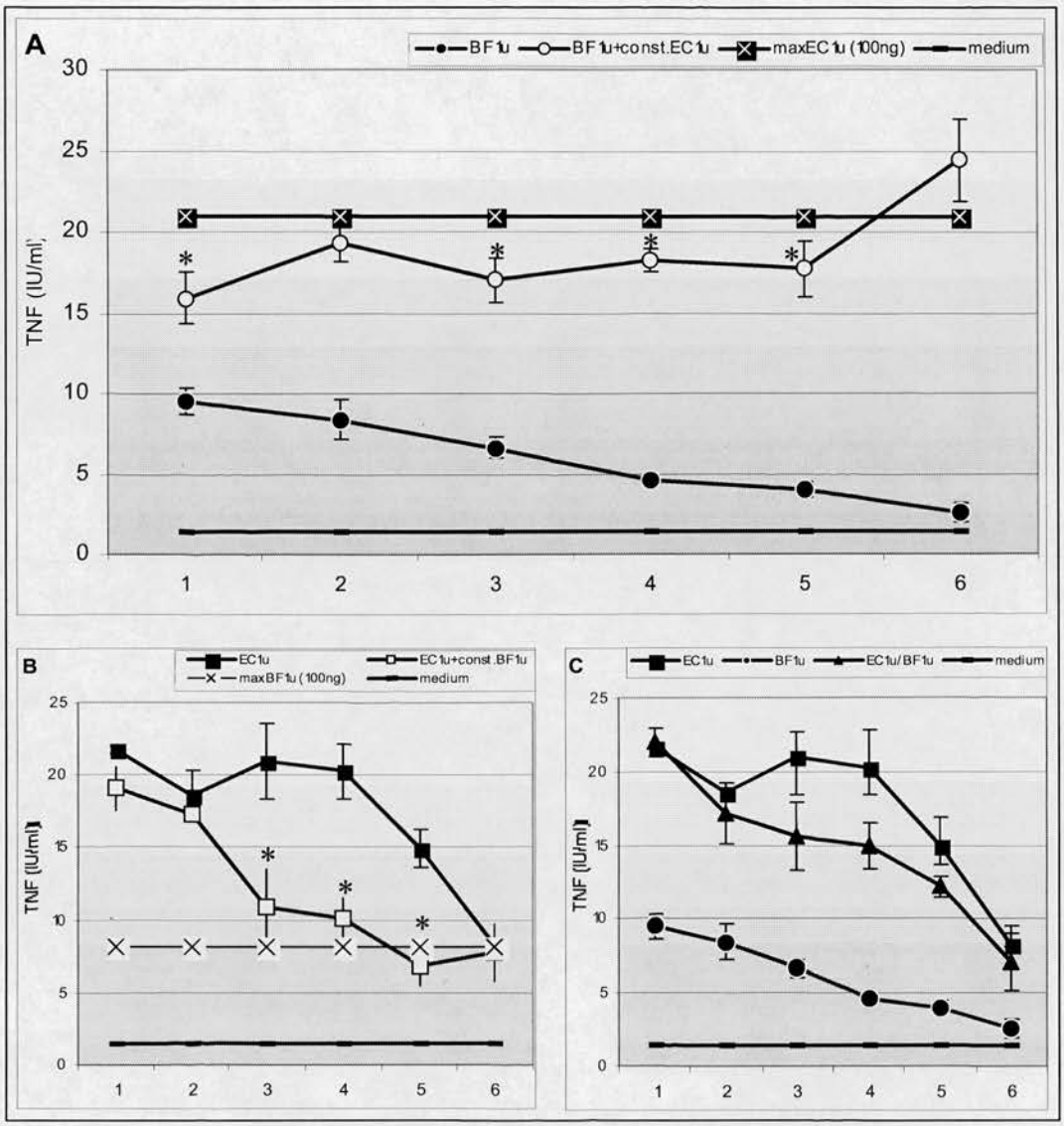

Figure 3.8 TNFα production (IU/ml) by human PBMC after interactions between EC1u and BF1u in different combinations and measured by ELISA

(A) Comparing between various concentrations of BF1u LPS alone and various concentrations of BF1u LPS plus constant concentration of EC1u (100ng/ml). (B) Comparing between various concentrations of EC1u LPS alone and various concentration of EC1u LPS plus constant concentration of BF1u (100ng/ml). (C) Comparing between various concentration of EC1u LPS alone, various concentrations of BF1u LPS alone and various mixtures of equal concentrations of EC1u and BF1u. Results represent the means +/- SEM for at least three experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

1= 1000ng/ml, 2=100ng/ml, 3=10ng/ml, 4=1ng/ml, 5=0.1ng/ml, 6=0.01ng/ml.

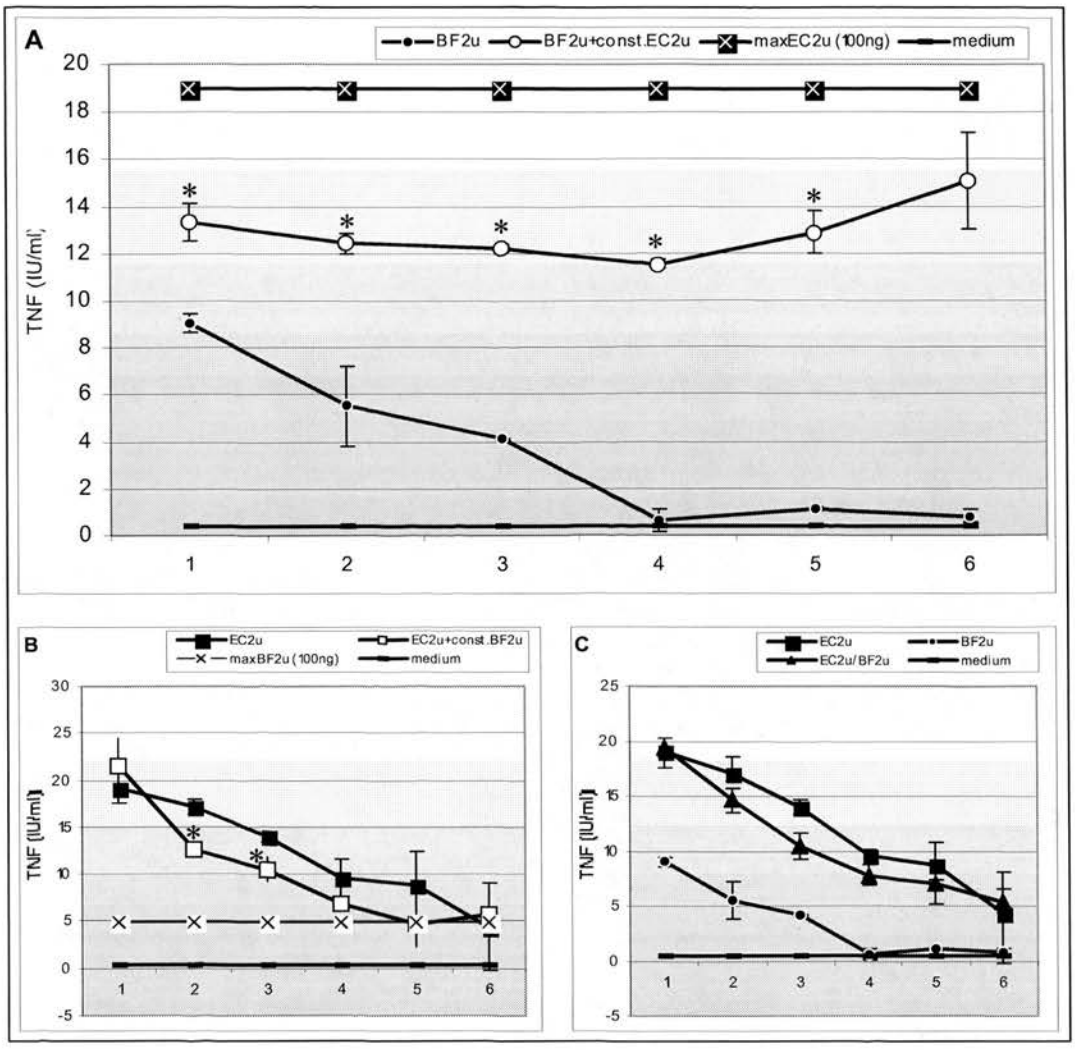

Figure 3.9 TNFα production (IU/ml) by human PBMC after interactions between EC2u and BF2u in different combinations and measured by ELISA

(A) Comparing between various concentrations of BF2u LPS alone and various concentrations of BF2u LPS plus constant concentration of EC2u (100ng/ml). (B) Comparing between various concentrations of EC2u LPS alone and various concentration of EC2u LPS plus constant concentration of BF2u (100ng/ml). (C) Comparing between various concentration of EC2u LPS alone, various concentrations of BF2u LPS alone and various mixtures of equal concentrations of EC2u and BF2u. Results represent the means +/- SEM for at least three experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

1= 1000ng/ml, 2=100ng/ml, 3=10ng/ml, 4=1ng/ml, 5=0.1ng/ml, 6=0.01ng/ml.

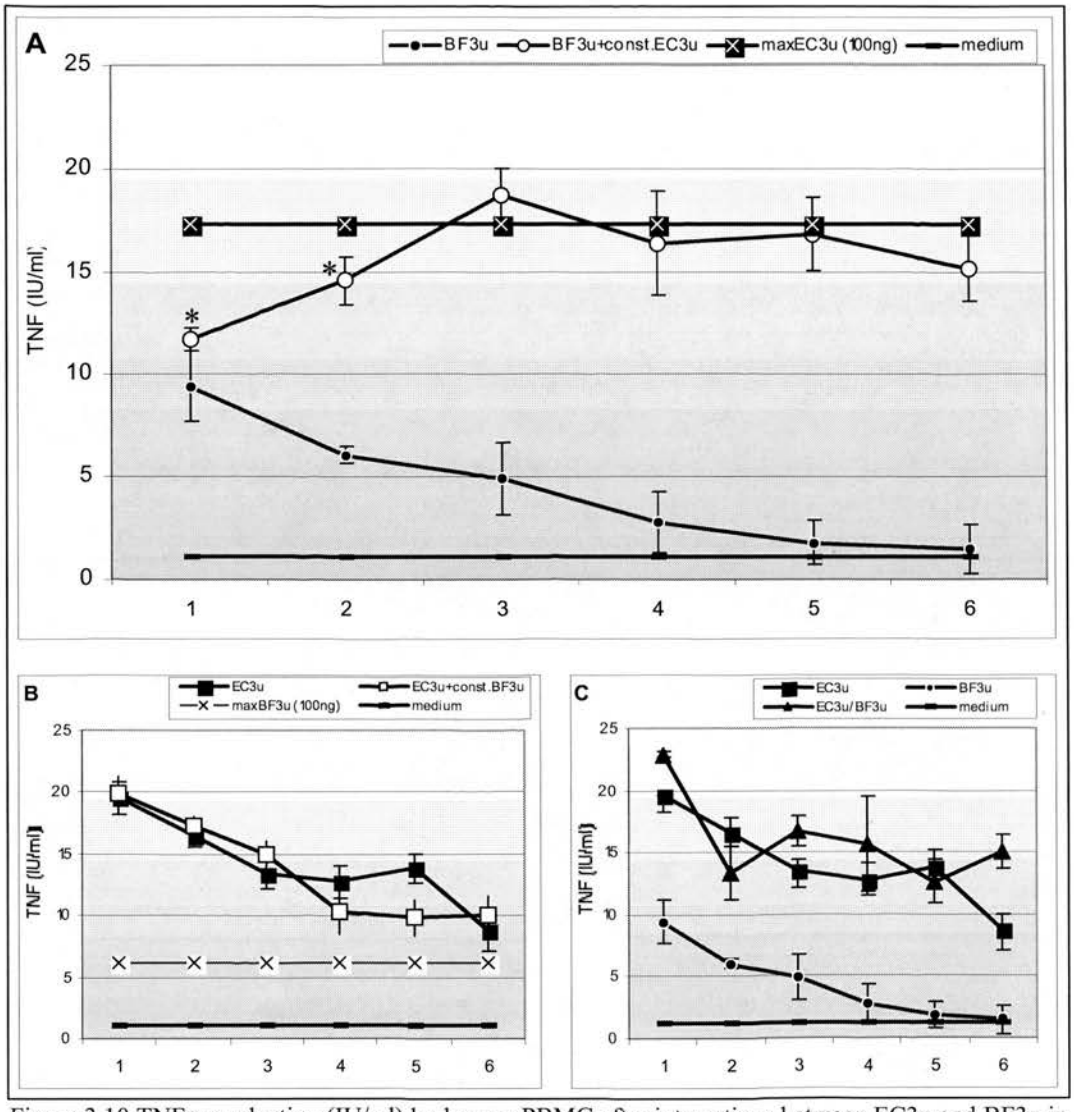

Figure 3.10 TNFα production (IU/ml) by human PBMC after interactions between EC3u and BF3u in different combinations and measured by ELISA

(A) Comparing between various concentrations of BF3u LPS alone and various concentrations of BF3u LPS plus constant concentration of EC3u (100ng/ml). (B) Comparing between various concentrations of EC3u LPS alone and various concentration of EC3u LPS plus constant concentration of BF3u (100ng/ml). (C) Comparing between various concentration of EC3u LPS alone, various concentrations of BF3u LPS alone and various mixtures of equal concentrations of EC3u and BF3u. Results represent the means +/- SEM for at least three experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

1= 1000ng/ml, 2=100ng/ml, 3=10ng/ml, 4=1ng/ml, 5=0.1ng/ml, 6=0.01ng/ml.

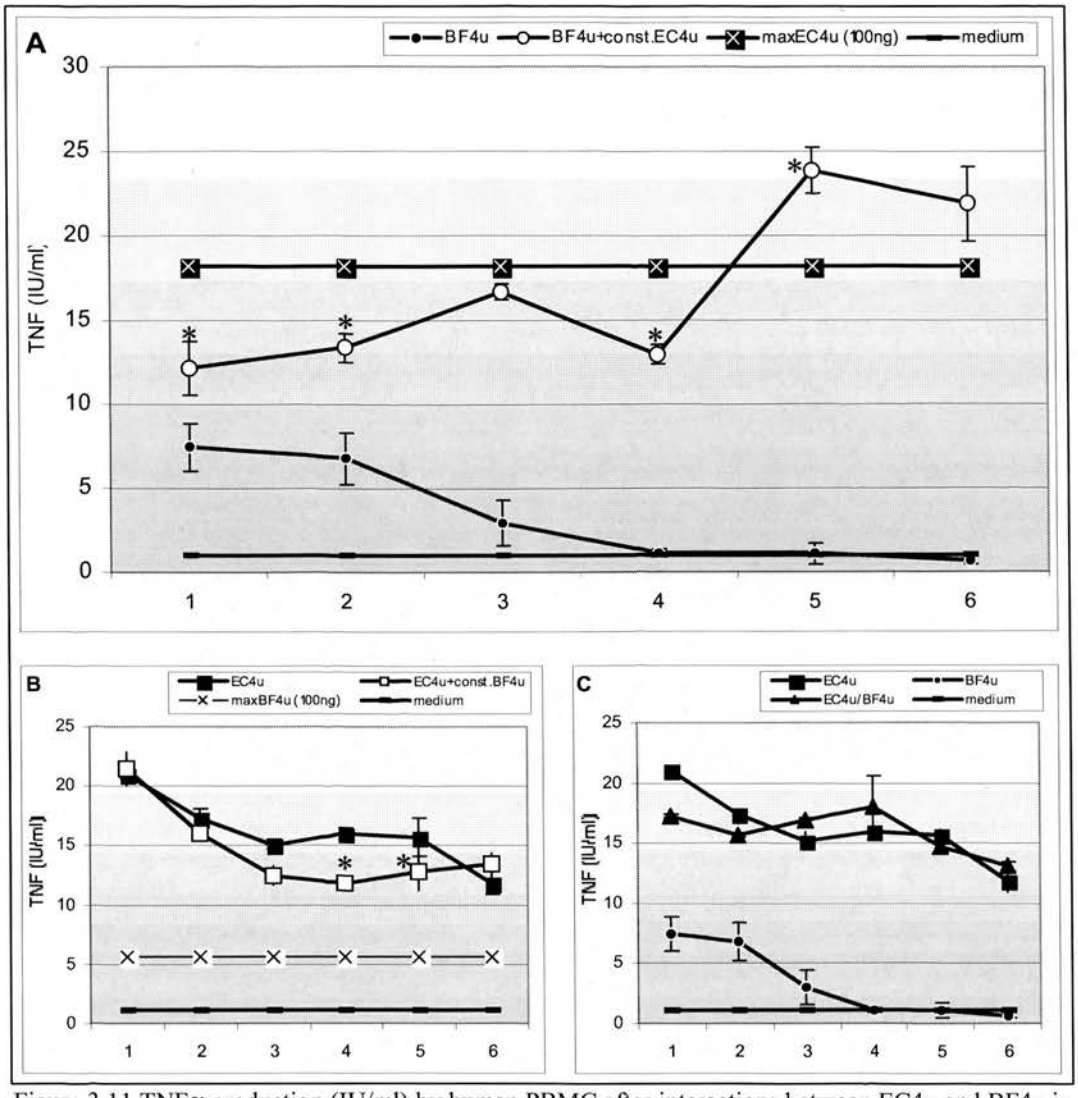

Figure 3.11 TNFα production (IU/ml) by human PBMC after interactions between EC4u and BF4u in different combinations and measured by ELISA

(A) Comparing between various concentrations of BF4u LPS alone and various concentrations of BF4u LPS plus constant concentration of EC4u (100ng/ml). (B) Comparing between various concentrations of EC4u LPS alone and various concentration of EC4u LPS plus constant concentration of BF4u (100ng/ml). (C) Comparing between various concentration of EC4u LPS alone, various concentrations of BF4u LPS alone and various mixtures of equal concentrations of EC4u and BF4u. Results represent the means +/- SEM for at least three experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

1= 1000ng/ml, 2=100ng/ml, 3=10ng/ml, 4=1ng/ml, 5=0.1ng/ml, 6=0.01ng/ml.

was able to exert a lowering effect against all of the *E. coli* LPS preparations to the extent of half of the TNF-α produced by 1μg/ml of EC1u, EC2u and EC4u LPSs alone although they were statistically significant with EC1u and EC4u LPSs. It also exerted a significant lowering effect against EC3u LPS when it went beyond this low level to almost a third of the amount produce by EC3u LPS alone. The effect of RS1 LPS against *B. fragilis* LPS preparations (Fig3.12B) was not to the same level compared with *E.coli* LPSs. There is very small non-significant lowering effect by RS1 against all except BF3u and BF2u. However, Fig 3.12C shows a noticeable lowering effect of RS1 LPS against all of the *Ps. aeruginosa* LPS preparations and it was significant with PA1u and PA3u LPSs which in presence of RS1 LPS produced third and half of the amount of TNF-α respectively.

Preparations of a small stock of *R. sphaeroides* LPSs were given by Mr R. Brown and Mr F. Pike. These LPS preparations were then examined for their ability to stimulate TNF-α producion and interact with one preparation of *E.coli* LPS (Fig 3.13). RS2 LPS was fully depigmented and extracted by PCP method with water, RS3 LPS was partially depigmented and extracted by PCP method with water and RS4 was not depigmented and extracted by PCP method with water. Fig 3.13A shows the TNF-α production after interactions between RS2, RS3 and RS4 at two concentrations, 10μg/ml and 100μg/ml, with EC1u at 1μg/ml after immediate mixing. As a rule, significant lowering effects of all of these different preparations of RS2, RS3 and RS4 were demonstrated with nothing much to distinguish between the two different concentrations of each of them except with RS4/EC1u in which the higher concentration of 100μg/ml led to a greater lowering effect against EC1u. Fig 3.13B shows the TNF-α production after interactions between RS2, RS3 and RS4 at two concentrations, 10μg/ml and 100μg/ml, which were added to the cells 2 hour before adding EC1u at 1μg/ml. Again significant lowering effect was also noticed with all *R. sphaeroides* LPS preparations ($p < 0.05$).

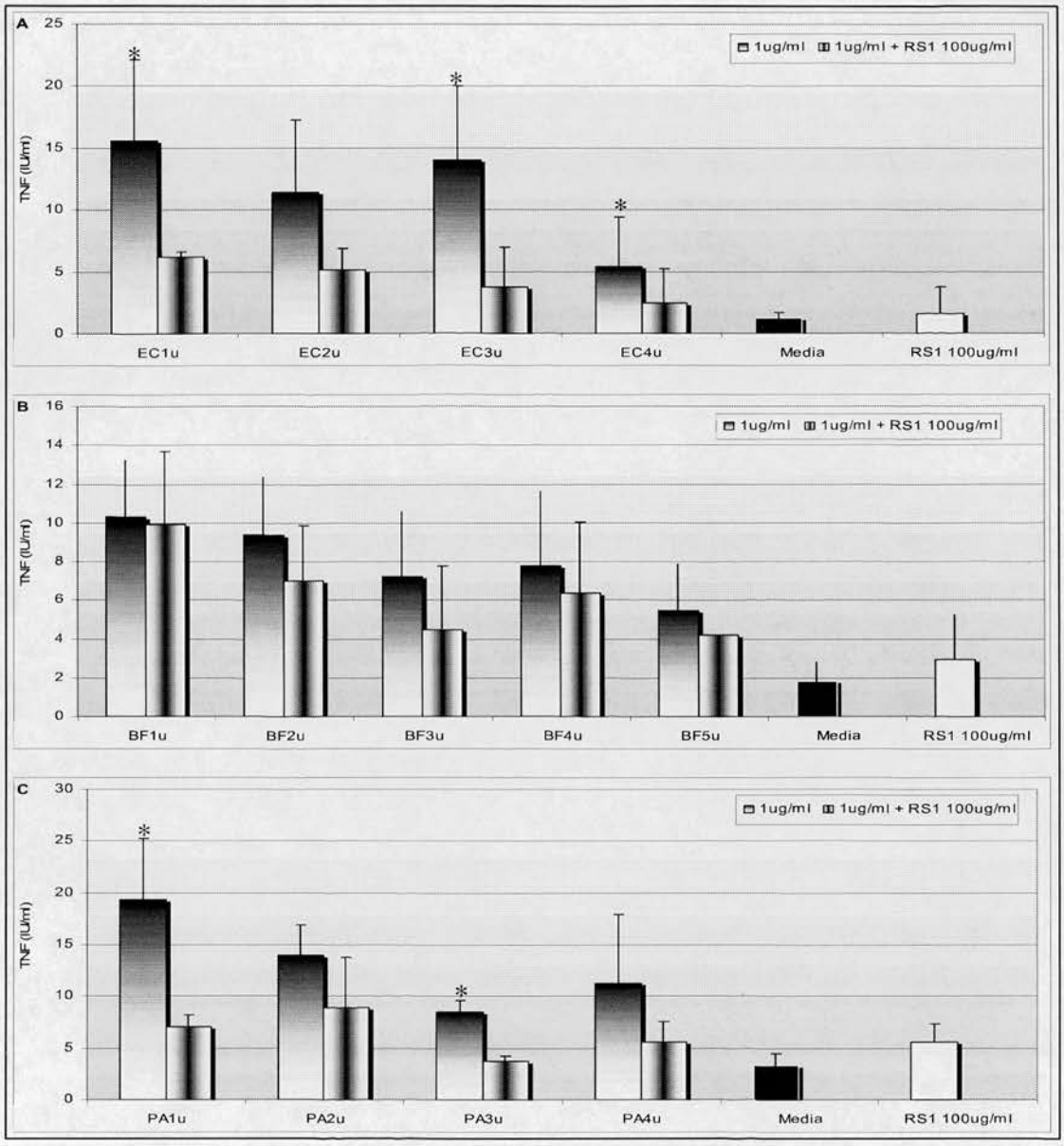

Figure 3.12 TNFα production (IU/ml) by human PBMC after interactions between different LPSs from different bacterial species and RS1 LPS and measured by L929 cells

(A) TNF-α production from different *E. coli* LPSs at 1μg/ml alone and mixed with *R. sphaeroides* LPS at 100μg/ml. (B) TNF-α production from different *B. fragilis* LPSs at 1μg/ml alone and mixed with *R. sphaeroides* LPS at 100μg/ml. (C) TNF-α production from different *Ps. aeruginosa* LPSs at 1μg/ml alone and mixed with *R. sphaeroides* LPS at 100μg/ml. (RS1) *R. sphaeroides* LPS fully depigmented and extracted by AP method. Results represent the means +/- SEM for at least three experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

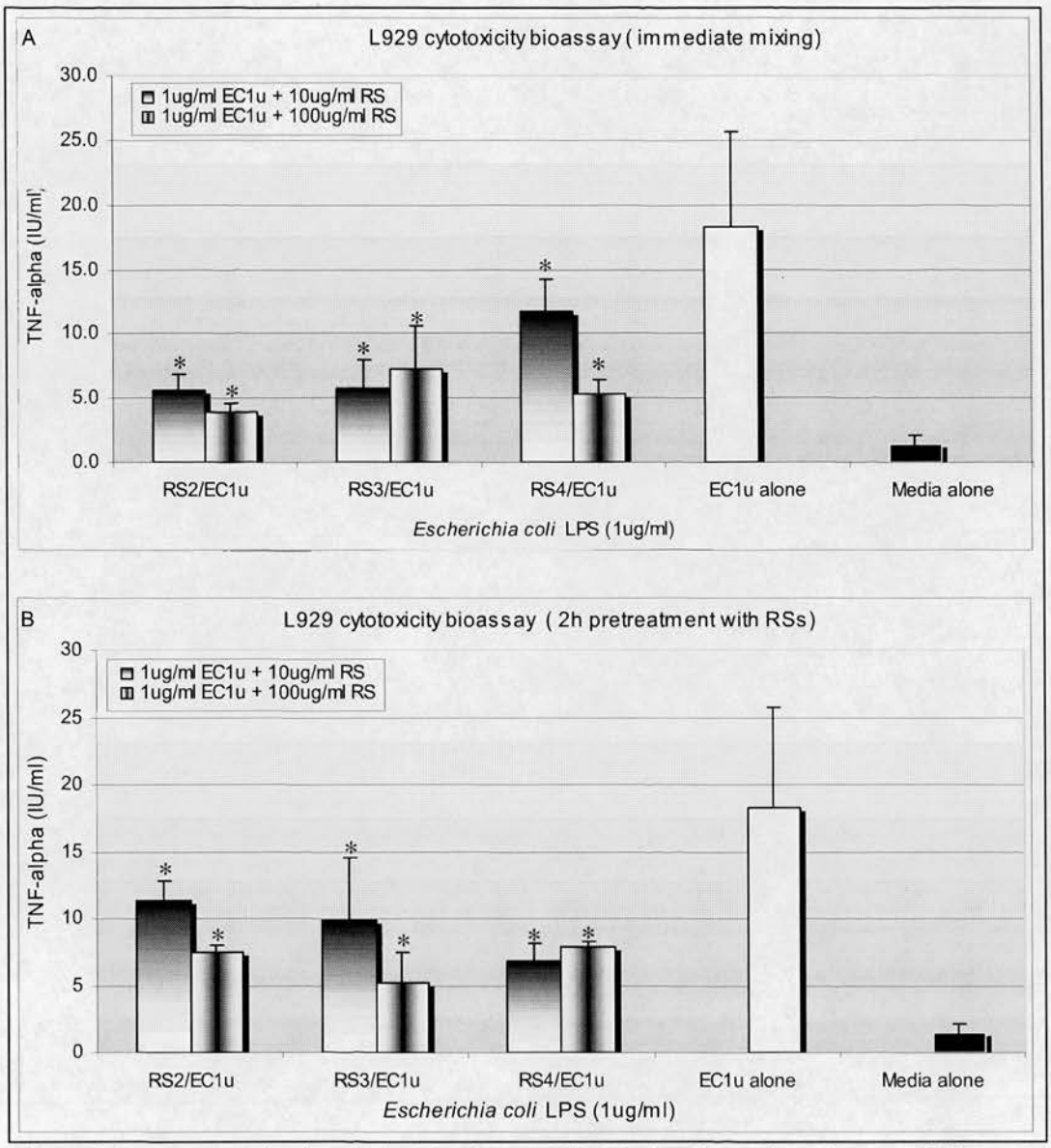

Figure 3.13 TNFα production (IU/ml) by human PBMC after interactions between different *R. sphaeroides* LPS preparations and *E. coli* LPS extracted by TMP method

(A) TNF-α production from different *R. sphaeroides* LPS preparations at two concentrations 10μg/ml and 100μg/ml mixed immediately with *E. coli* LPS of EC1u at 1μg/ml. (B) TNF-α production from different *R. sphaeroides* LPS preparations at two concentrations 10μg/ml and 100μg/ml which added first to the human PBMC alone and after 2 hour of pre-treatment an *E. coli* LPS of EC1u at 1μg/ml were then mixed with. The TNFα production was measured by L929 cells. (RS2) *R. sphaeroides* LPS fully depigmented and extracted by PCP method. (RS3) *R. sphaeroides* LPS partially depigmented and extracted by PCP method (RS4) *R. sphaeroides* LPS not depigmented and extracted by PCP method. Results represent the means +/- SEM for at least three experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

3.5 Interactions between different LPSs from different bacterial species in stimulation TNF-α production by THP-1 cell line

All the time, at least two plates of Vitamin D3 treated and untreated THP-1 cells were prepared for the interactions experiment.

3.5.1 Interactions between EC1u and BF1u LPSs on TNF-α production and measured by L929 cell cytotoxicity assay

Using the L929 assay to measure the TNF-α production after challenging THP-1 cells, interaction approaches were done between EC1u and BF1u LPSs in the manner identical to that described in Section 3.4.1. Fig3.14 represented THP-1 without treatment of Vitamin D3. Fig 3.14 A shows the lowering effect of the various concentrations of BF1u against a constant concentration of EC1u, 100ng/ml, at all concentrations except the lowest one of BF1u. This lowering effect was statistically significant at 100, 10, 1 and 0.1ngs. In Fig 3.14B, EC1u+constBF1u was significantly higher than maxBF2u at the first three higher concentrations while there was no significant differences between EC1u+constBF1u and EC1u at any concentration. Again Fig 3.14C shows no significant differences between EC1u/BF1u and EC1u at any concentration although TNF-α production in response to EC1u/BF1u was significantly higher than that of BF1u alone ($p<0.05$).

Where Vitamin D3 was included in the incubation with THP-1 cells (Fig 3.15) the BF1u still inhibited TNF-α production at the three highest concentrations used but this lowering effect was statistically significant only at 100ng as shown in Fig3.15A. Fig 3.14A shows more significant lowering effect than it was shown in Fig 3.15A. When the conditions are reversed, using constant concentration of BF1u LPS at 100ng/ml as shown in Fig 3.15B, no significant differences was calculated at any concentrations. However, EC1u+constBF1u was significantly higher than maxBF1u at the first three higher concentrations. The general lowering effect is again demonstrated although the effect is less marked. In Fig3.15C, no significant differences were calculated between EC1u/BF1u and EC1u. Nevertheless, EC1u/BF1u was significantly higher than BF1u ($p<0.05$).

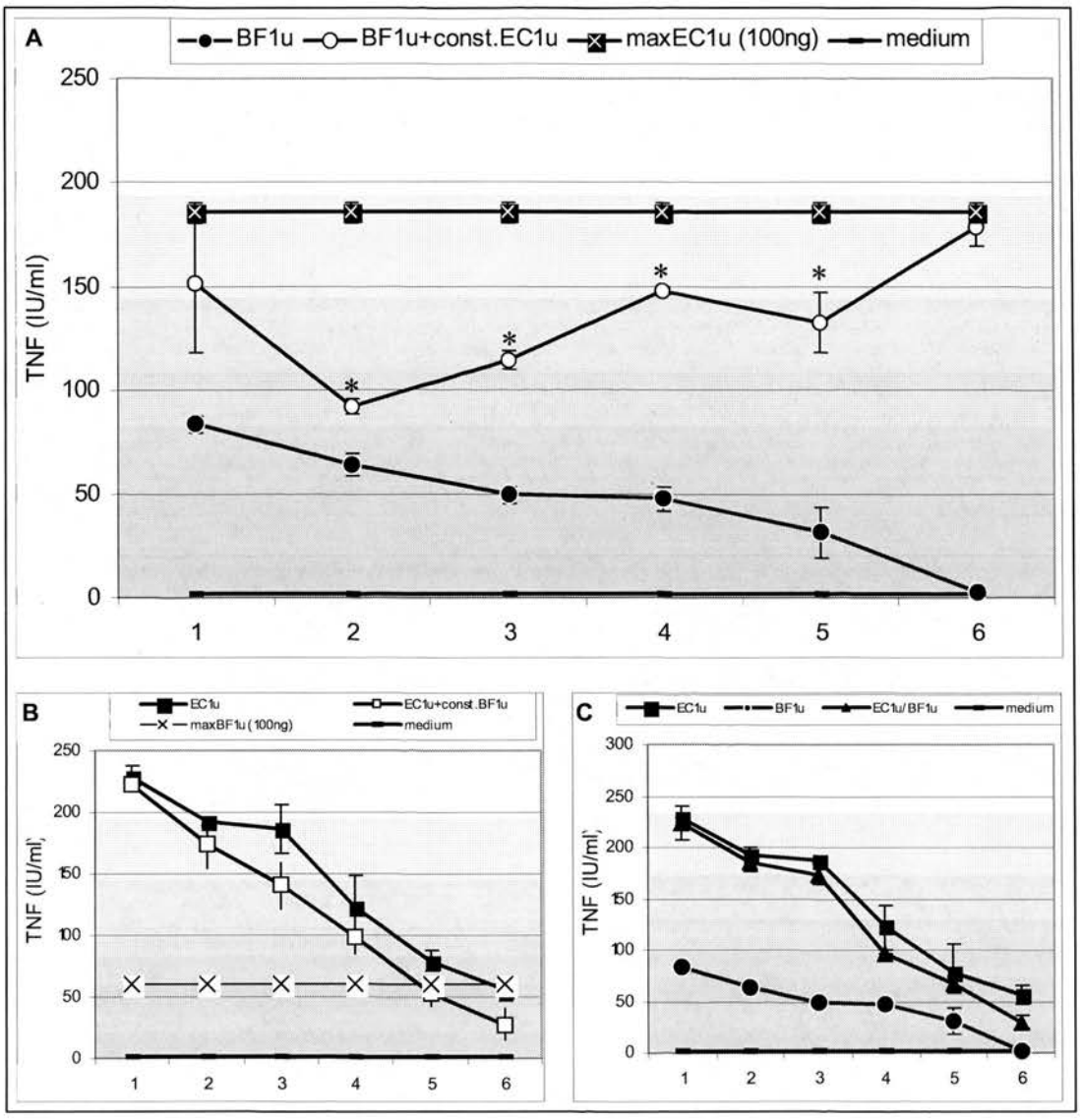

Figure 3.14 TNFα production (IU/ml) by THP-1 cell without VD3 treatment after interactions between EC1u and BF1u in different combinations and measured by L929

(A) Comparing between various concentrations of BF1u LPS alone and various concentrations of BF1u LPS plus constant concentration of EC1u (100ng/ml). (B) Comparing between various concentrations of EC1u LPS alone and various concentration of EC1u LPS plus constant concentration of BF1u (100ng/ml). (C) Comparing between various concentration of EC1u LPS alone, various concentrations of BF1u LPS alone and various mixtures of equal concentrations of EC1u and BF1u. Results represent the means +/- SEM for at least three experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

1= 1000ng/ml, 2=100ng/ml, 3=10ng/ml, 4=1ng/ml, 5=0.1ng/ml, 6=0.01ng/ml.

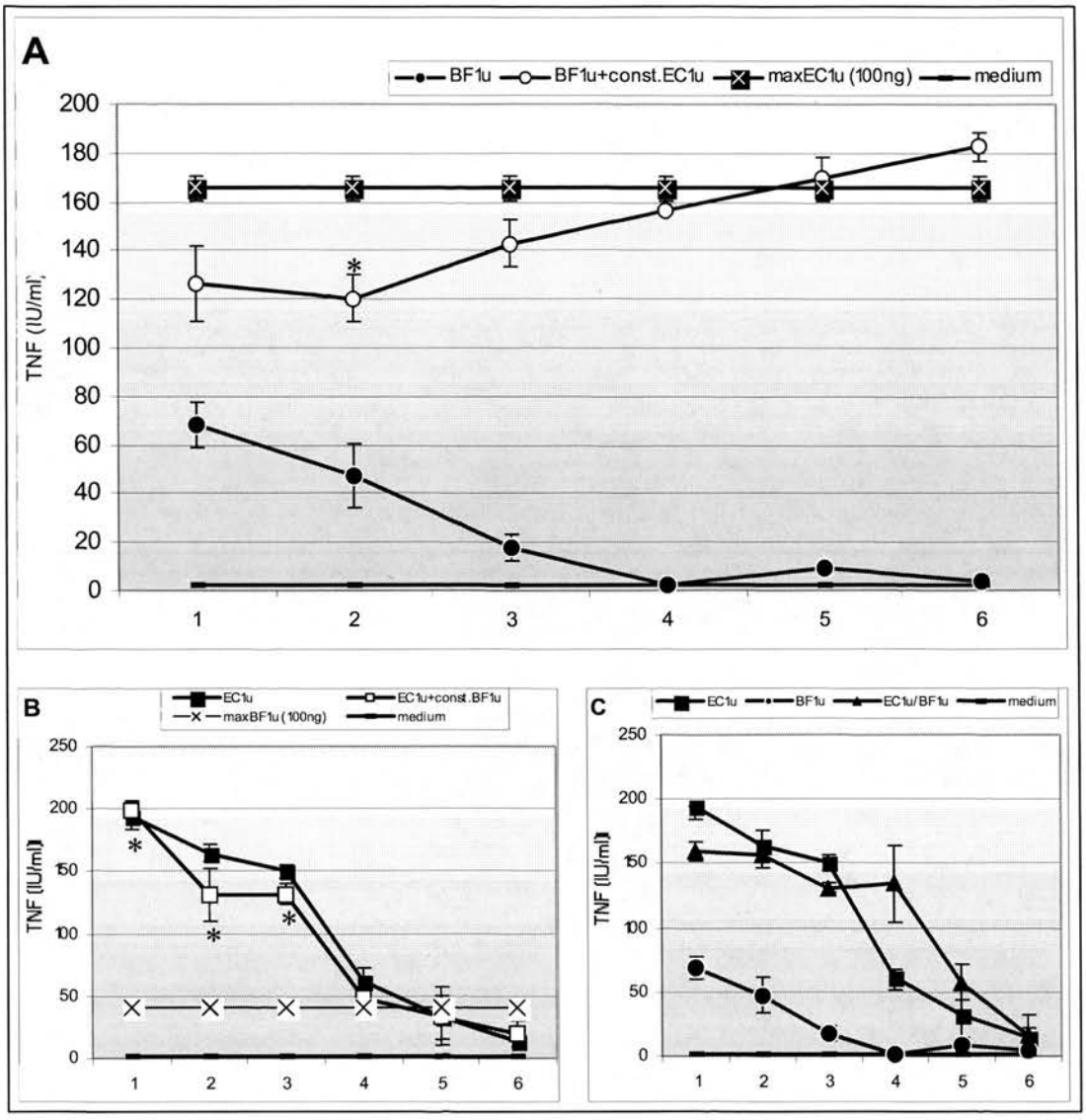

Figure 3.15 TNFα production (IU/ml) by THP-1 cell with VD3 treatment after interactions between EC1u and BF1u in different combinations and measured by L929

(A) Comparing between various concentrations of BF1u LPS alone and various concentrations of BF1u LPS plus constant concentration of EC1u (100ng/ml). (B) Comparing between various concentrations of EC1u LPS alone and various concentration of EC1u LPS plus constant concentration of BF1u (100ng/ml). (C) Comparing between various concentration of EC1u LPS alone, various concentrations of BF1u LPS alone and various mixtures of equal concentrations of EC1u and BF1u. Results represent the means +/- SEM for at least three experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

1= 1000ng/ml, 2=100ng/ml, 3=10ng/ml, 4=1ng/ml, 5=0.1ng/ml, 6=0.01ng/ml.

3.5.2 Interactions between EC2u and BF2u LPSs on TNF-α production and measured by L929 cell cytotoxicity assay

Fig 3.16A and Fig3.17A demonstrate the lowering effect of BF2u LPS against constant concentration of EC2u LPS at 100ng/ml. This effect is totally reduced at the two lowest concentrations 0.1ng/ml and 0.01ng/ml. In Fig 3.16A, the lowering effect of BF2u LPS was statistically significant compared to constant EC2u at the first three higher concentrations. Although there was no significant differences between EC2u and EC2u+constBF2u at any concentration (Fig 3.16B), TNF-α response by EC2u+constBF2u was significantly higher than that of maxBF2u. In Fig 3.16C, EC2u/BF2u shows significant differences compared to BF2u at the first three higher concentrations. EC2u/BF2u also showed significant differences compared to EC2u at the three lower concentrations ($p<0.05$).

Similar to what shown in Fig 3.16A, significant lowering effect was noticed at 100, 10 and 1ngs of BF2u+constEC2u compared to maxEC2u inFig3.17A where THP-1 cells were treated by Vitamin D3. In Fig 3.17B, no significant differences was shown between EC2u+const BF2u and EC2u except at 0.01ng. On the other hand, TNF-α response by EC2u+const BF2u was significantly higher than that of maxBF2u at the first three higher concentrations. Fig3.17C shows no significant differences between EC2u/BF2u and EC2u at any concentration. Although the same figure shows significant differences between EC2u/BF2u and BF2u at all concentrations ($p<0.05$).

3.5.3 Interactions between EC3u and BF3u LPSs on TNF-α production and measured by L929 cell cytotoxicity assay

Fig 3.18A demonstrates a slightly fluctuating lowering effect of BF3u LPS against constant concentration of EC3u LPS at 100ng/ml. This effect was significant at 1000 and 10ng/ml but it almost non-existent at 1ng/ml and 0.1ng/ml concentrations. No significant differences between different combinations were noticed either in Fig 3.18B or in Fig 3.18C.

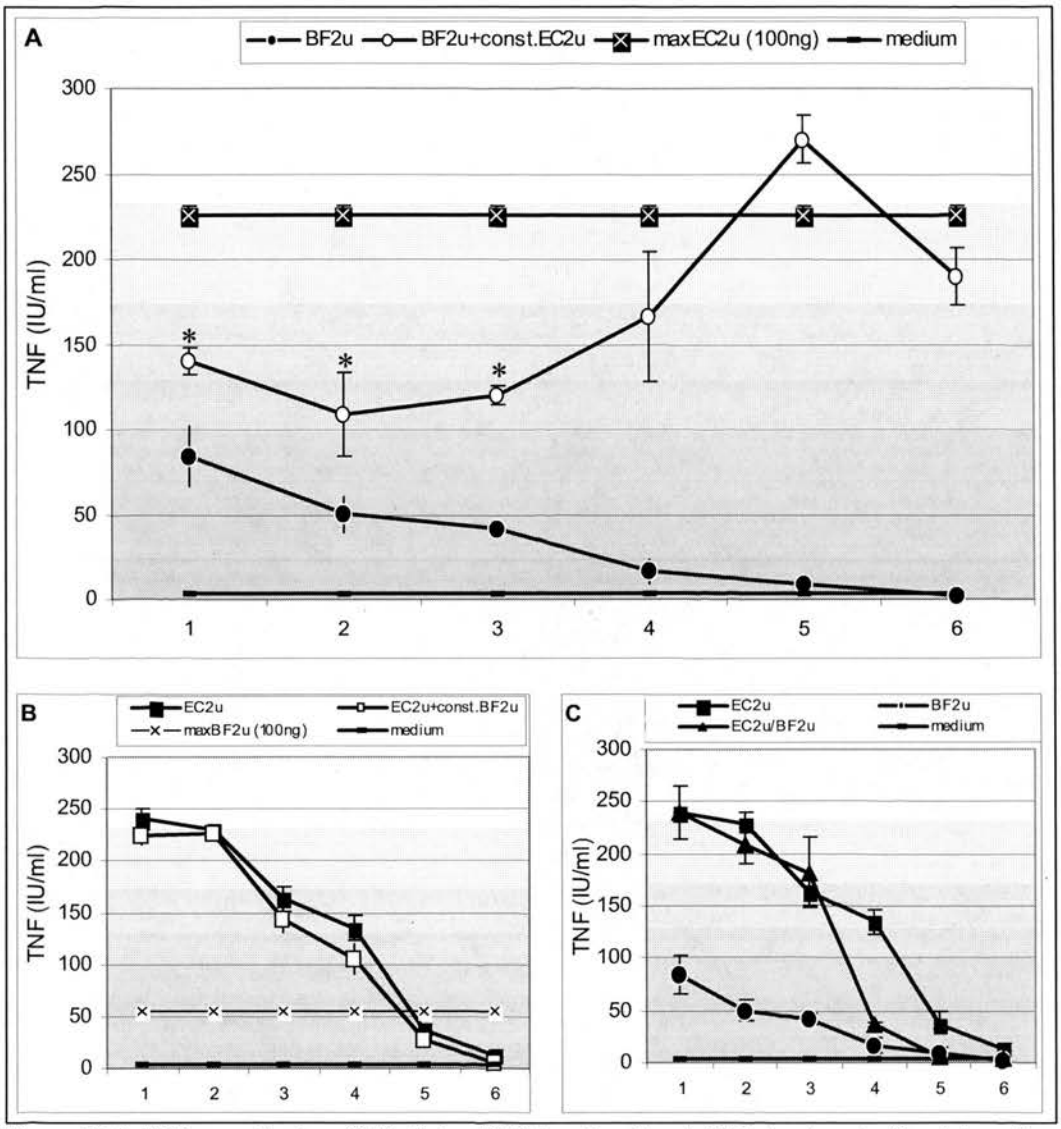

Figure 3.16 TNFα production (IU/ml) by THP-1 cell without VD3 treatment after interactions between EC2u and BF2u in different combinations and measured by L929

(A) Comparing between various concentrations of BF2u LPS alone and various concentrations of BF2u LPS plus constant concentration of EC2u (100ng/ml). (B) Comparing between various concentrations of EC2u LPS alone and various concentration of EC2u LPS plus constant concentration of BF2u (100ng/ml). (C) Comparing between various concentration of EC2u LPS alone, various concentrations of BF2u LPS alone and various mixtures of equal concentrations of EC2u and BF2u. Results represent the means +/- SEM for at least three experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

1= 1000ng/ml, 2=100ng/ml, 3=10ng/ml, 4=1ng/ml, 5=0.1ng/ml, 6=0.01ng/ml.

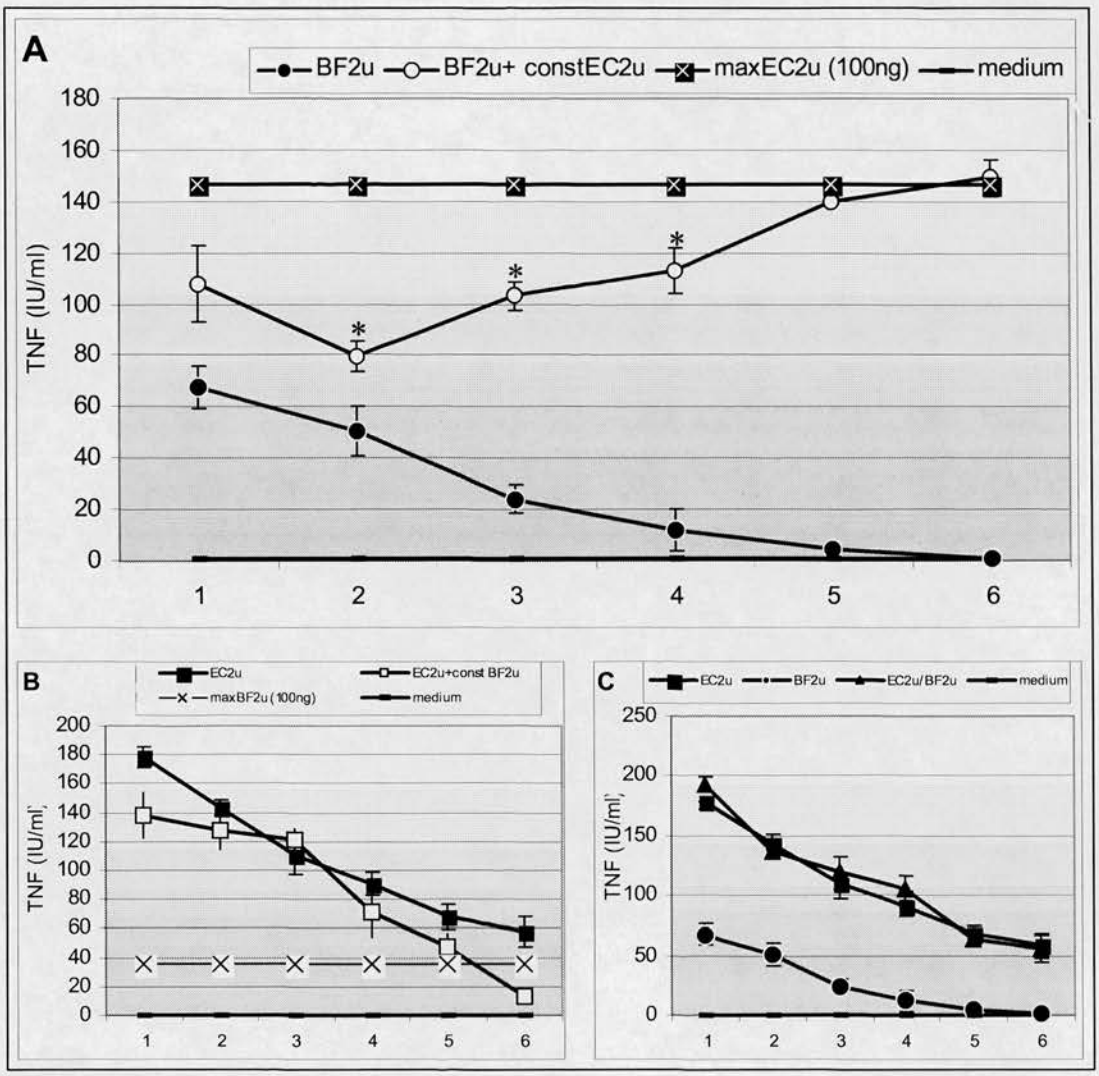

Figure 3.17 TNFα production (IU/ml) by THP-1 cell with VD3 treatment after interactions between EC2u and BF2u in different combinations and measured by L929

(A) Comparing between various concentrations of BF2u LPS alone and various concentrations of BF2u LPS plus constant concentration of EC2u (100ng/ml). (B) Comparing between various concentrations of EC2u LPS alone and various concentration of EC2u LPS plus constant concentration of BF2u (100ng/ml). (C) Comparing between various concentration of EC2u LPS alone, various concentrations of BF2u LPS alone and various mixtures of equal concentrations of EC2u and BF2u. Results represent the means +/- SEM for at least three experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

1= 1000ng/ml, 2=100ng/ml, 3=10ng/ml, 4=1ng/ml, 5=0.1ng/ml, 6=0.01ng/ml.

On the other hand, when Vitamin D3 treatment was included Fig 3.19A shows a dose-dependent lowering effect that completely vanished at the two lowest concentrations 0.1ng/ml and 0.01ng/ml. Although it was statistically significant only at the two higher concentrations. Fig 3.19B shows an obvious lowering effect of constant BF3u LPSs at all concentrations of EC3u LPS although it was significant only at 100 and 1ngs. No steady patterm can be deduced from the combinations shown in Fig3.19C ($p<0.05$).

3.5.4 Interactions between EC4u and BF4u LPSs on TNF-α production and measured by L929 cell cytotoxicity assay

Fig 3.20A revealed an obvious lowering effect of BF4u LPS against constant EC4u LPS at the two highest concentrations and two lower concentrations but not that much at 10ng/ml and 1ng/ml of BF4u LPS. This lowering effect was statistically significant at 1000, 0.1 and 0.01ngs. When THP-1 cells were treated by Vitamin D3, Fig 3.21A shows a fluctuating lowering effect of BF4u LPS against constant concentration of EC4u LPS. Although this effect is significant at 1000, 10 and 1ngs, it was not observed at 0.1ng/ml and 0.01ng/ml. Although Fig 3.20B and Fig 3.21B demonstrate a slight lowering effect exerted by constant amount of BF4u, no statistical differences were noticed in both figures. In Fig 3.20C, EC4u/BF4u showed a significant difference compared to both EC4u and BF4u. While Fig 3.21C shows no significant difference except between EC4u/BF4u and BF4u at the three higher concentrations ($p<0.05$).

3.5.5 Interactions between EC1u and BF1u LPSs on TNF-α production and measured by ELISA

Fig 3.22A shows a significant lowering effect of BF1u LPS against constant EC1u LPS at the three highest concentrations. This lowering effect was not seen at the three lowest concentrations. Significant differences were neither shown between EC1u+constBF1u and EC1u (Fig 3.22B) nor between EC1u/BF1u and EC1u at any concentrations ($p<0.05$).

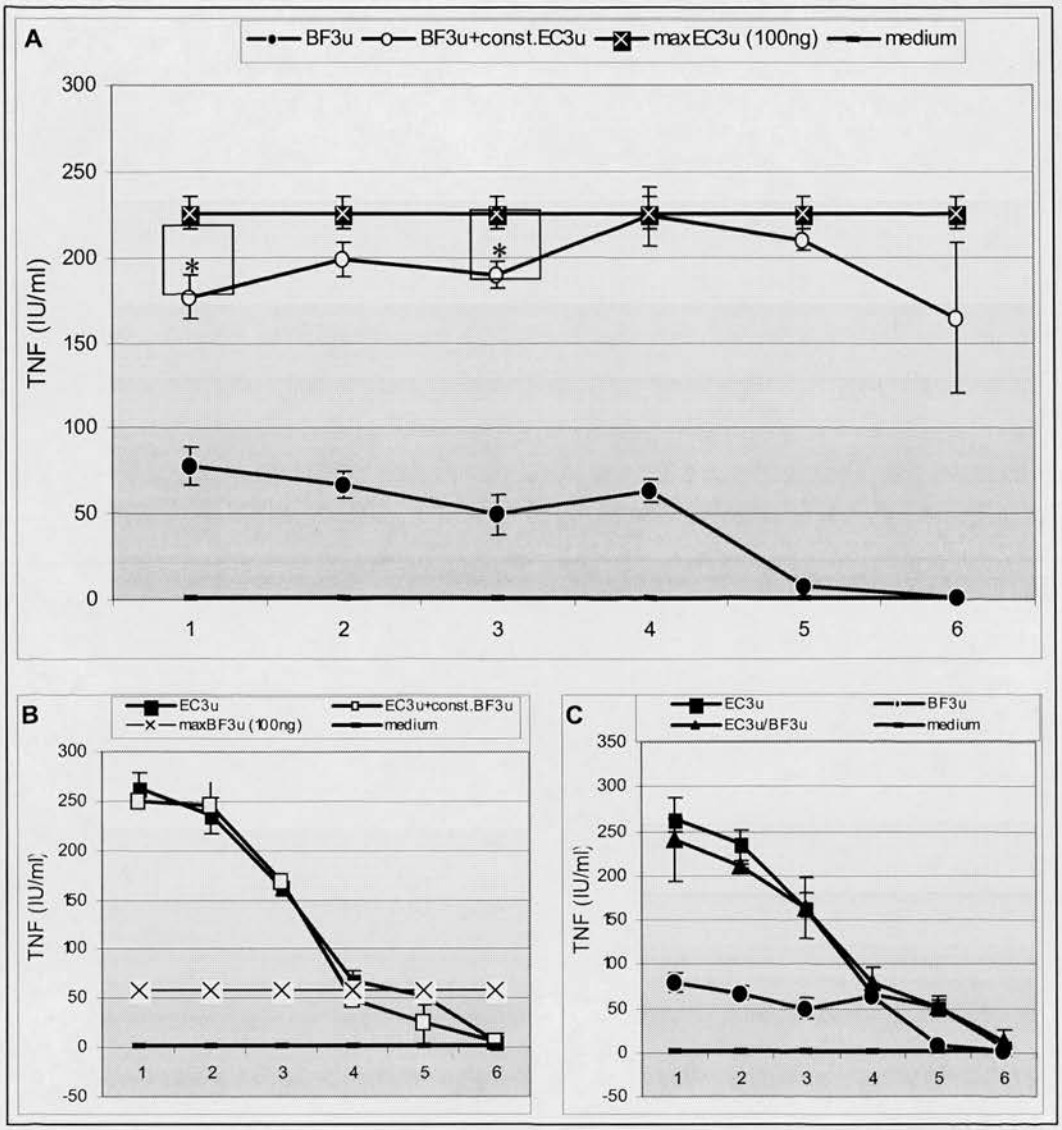

Figure 3.18 TNFα production (IU/ml) by THP-1 cell without VD3 treatment after interactions between EC3u and BF3u in different combinations and measured by L929

(A) Comparing between various concentrations of BF3u LPS alone and various concentrations of BF3u LPS plus constant concentration of EC3u (100ng/ml). (B) Comparing between various concentrations of EC3u LPS alone and various concentration of EC3u LPS plus constant concentration of BF3u (100ng/ml). (C) Comparing between various concentration of EC3u LPS alone, various concentrations of BF3u LPS alone and various mixtures of equal concentrations of EC3u and BF3u. Results represent the means +/- SEM for at least three experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

1= 1000ng/ml, 2=100ng/ml, 3=10ng/ml, 4=1ng/ml, 5=0.1ng/ml, 6=0.01ng/ml.

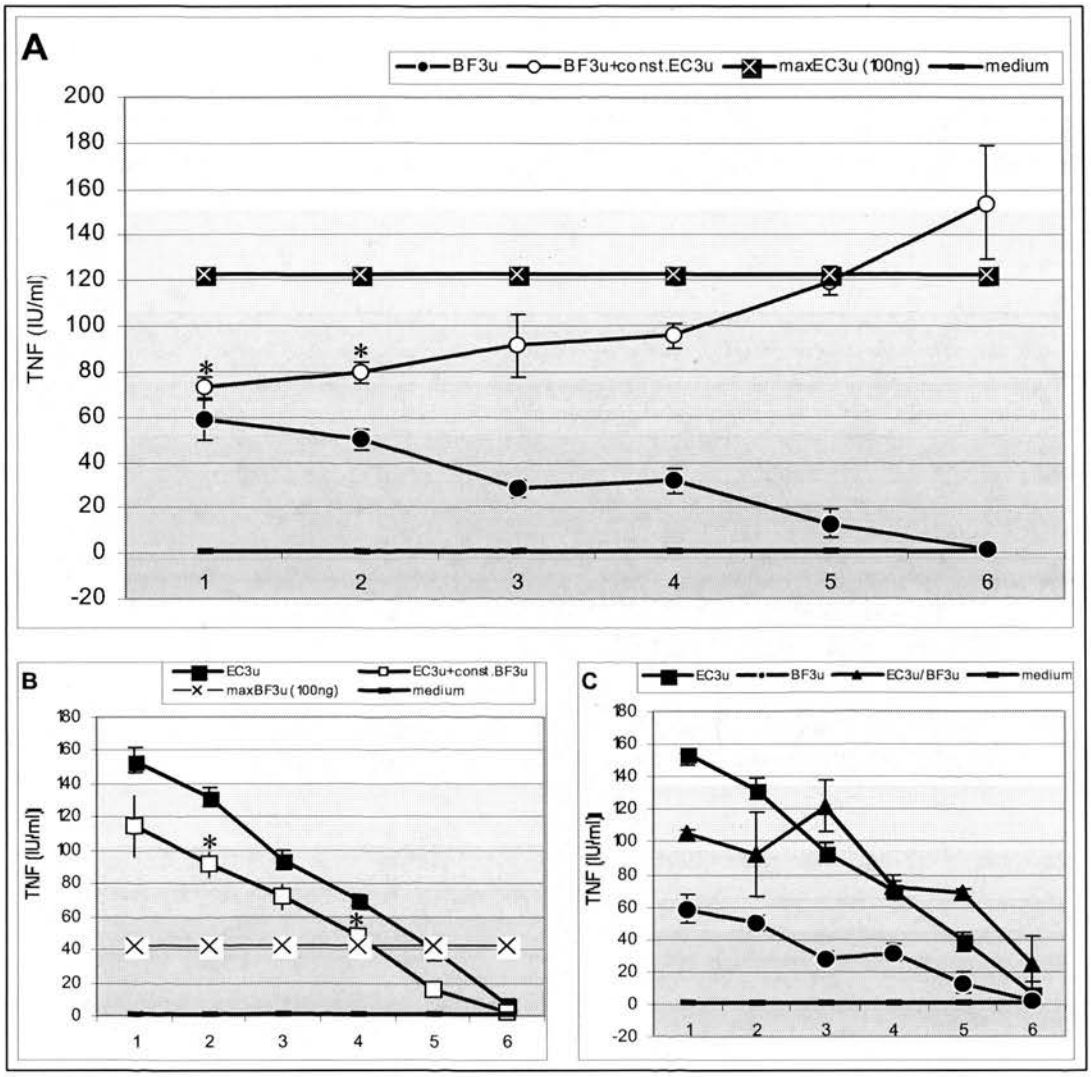

Figure 3.19 TNFα production (IU/ml) by THP-1 cell with VD3 treatment after interactions between EC3u and BF3u in different combinations and measured by L929

(A) Comparing between various concentrations of BF3u LPS alone and various concentrations of BF3u LPS plus constant concentration of EC3u (100ng/ml). (B) Comparing between various concentrations of EC3u LPS alone and various concentration of EC3u LPS plus constant concentration of BF3u (100ng/ml). (C) Comparing between various concentration of EC3u LPS alone, various concentrations of BF3u LPS alone and various mixtures of equal concentrations of EC3u and BF3u. Results represent the means +/- SEM for at least three experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

1= 1000ng/ml, 2=100ng/ml, 3=10ng/ml, 4=1ng/ml, 5=0.1ng/ml, 6=0.01ng/ml.

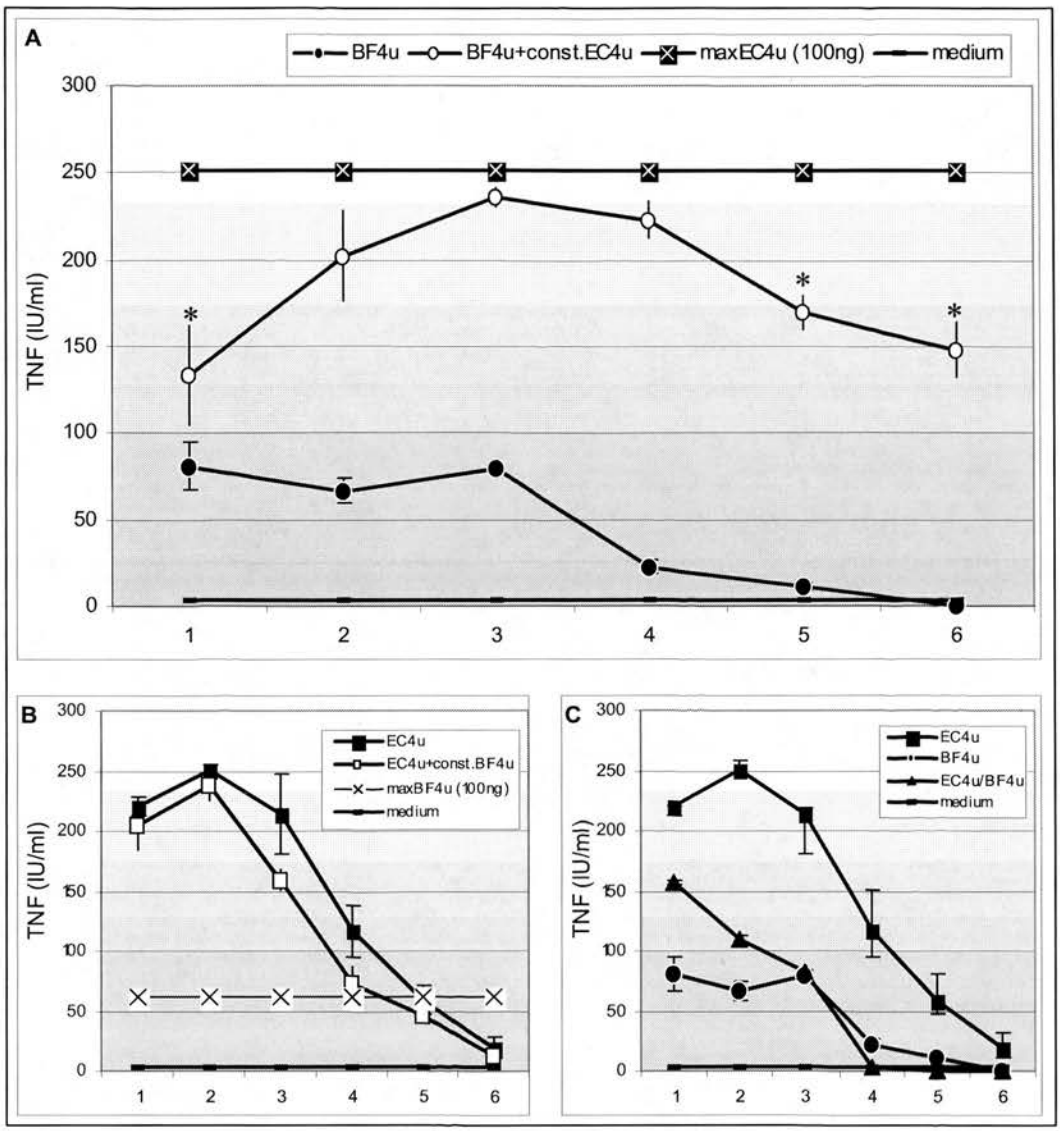

Figure 3.20 TNFα production (IU/ml) by THP-1 cell without VD3 treatment after interactions between EC4u and BF4u in different combinations and measured by L929

(A) Comparing between various concentrations of BF4u LPS alone and various concentrations of BF4u LPS plus constant concentration of EC4u (100ng/ml). (B) Comparing between various concentrations of EC4u LPS alone and various concentration of EC4u LPS plus constant concentration of BF4u (100ng/ml). (C) Comparing between various concentration of EC4u LPS alone, various concentrations of BF4u LPS alone and various mixtures of equal concentrations of EC4u and BF4u. Results represent the means +/- SEM for at least three experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

1= 1000ng/ml, 2=100ng/ml, 3=10ng/ml, 4=1ng/ml, 5=0.1ng/ml, 6=0.01ng/ml.

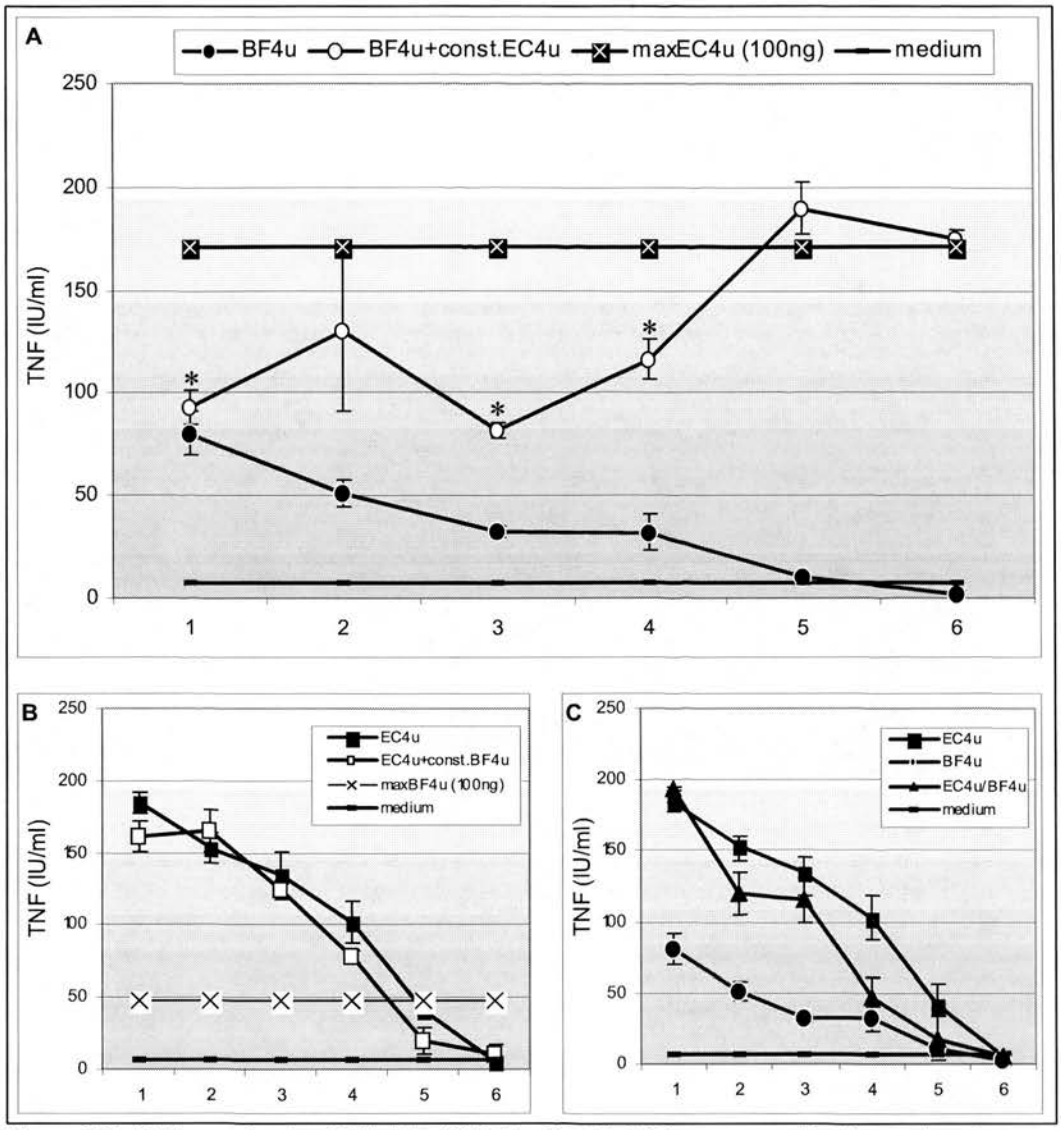

Figure 3.21 TNFα production (IU/ml) by THP-1 cell with VD3 treatment after interactions between EC4u and BF4u in different combinations and measured by L929

(A) Comparing between various concentrations of BF4u LPS alone and various concentrations of BF4u LPS plus constant concentration of EC4u (100ng/ml). (B) Comparing between various concentrations of EC4u LPS alone and various concentration of EC4u LPS plus constant concentration of BF4u (100ng/ml). (C) Comparing between various concentration of EC4u LPS alone, various concentrations of BF4u LPS alone and various mixtures of equal concentrations of EC4u and BF4u. Results represent the means +/- SEM for at least three experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

1= 1000ng/ml, 2=100ng/ml, 3=10ng/ml, 4=1ng/ml, 5=0.1ng/ml, 6=0.01ng/ml.

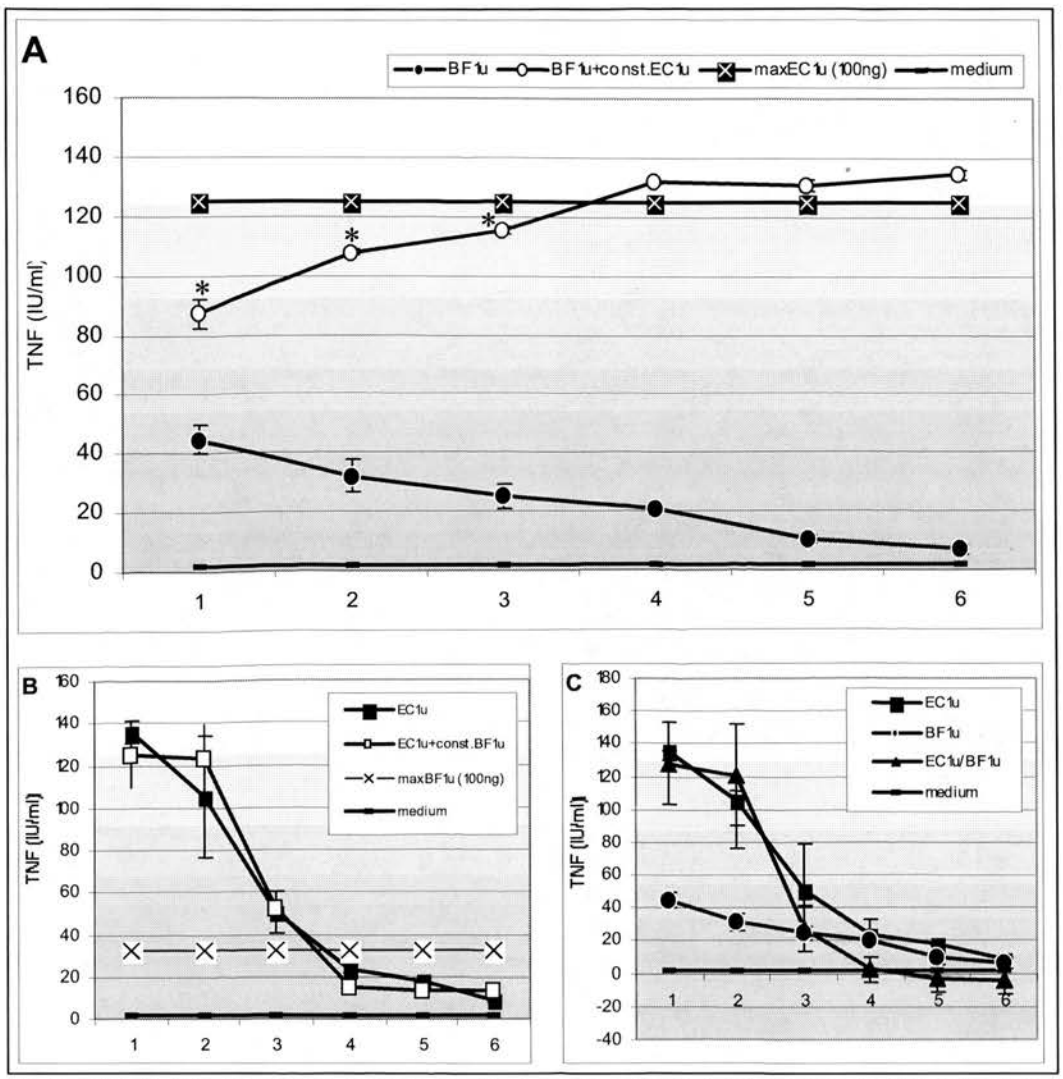

Figure 3.22 TNFα production (IU/ml) by THP-1 cell without VD3 treatment after interactions between EC1u and BF1u in different combinations and measured by ELISA

(A) Comparing between various concentrations of BF1u LPS alone and various concentrations of BF1u LPS plus constant concentration of EC1u (100ng/ml). (B) Comparing between various concentrations of EC1u LPS alone and various concentration of EC1u LPS plus constant concentration of BF1u (100ng/ml). (C) Comparing between various concentration of EC1u LPS alone, various concentrations of BF1u LPS alone and various mixtures of equal concentrations of EC1u and BF1u. Results represent the means +/- SEM for at least three experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

1= 1000ng/ml, 2=100ng/ml, 3=10ng/ml, 4=1ng/ml, 5=0.1ng/ml, 6=0.01ng/ml.

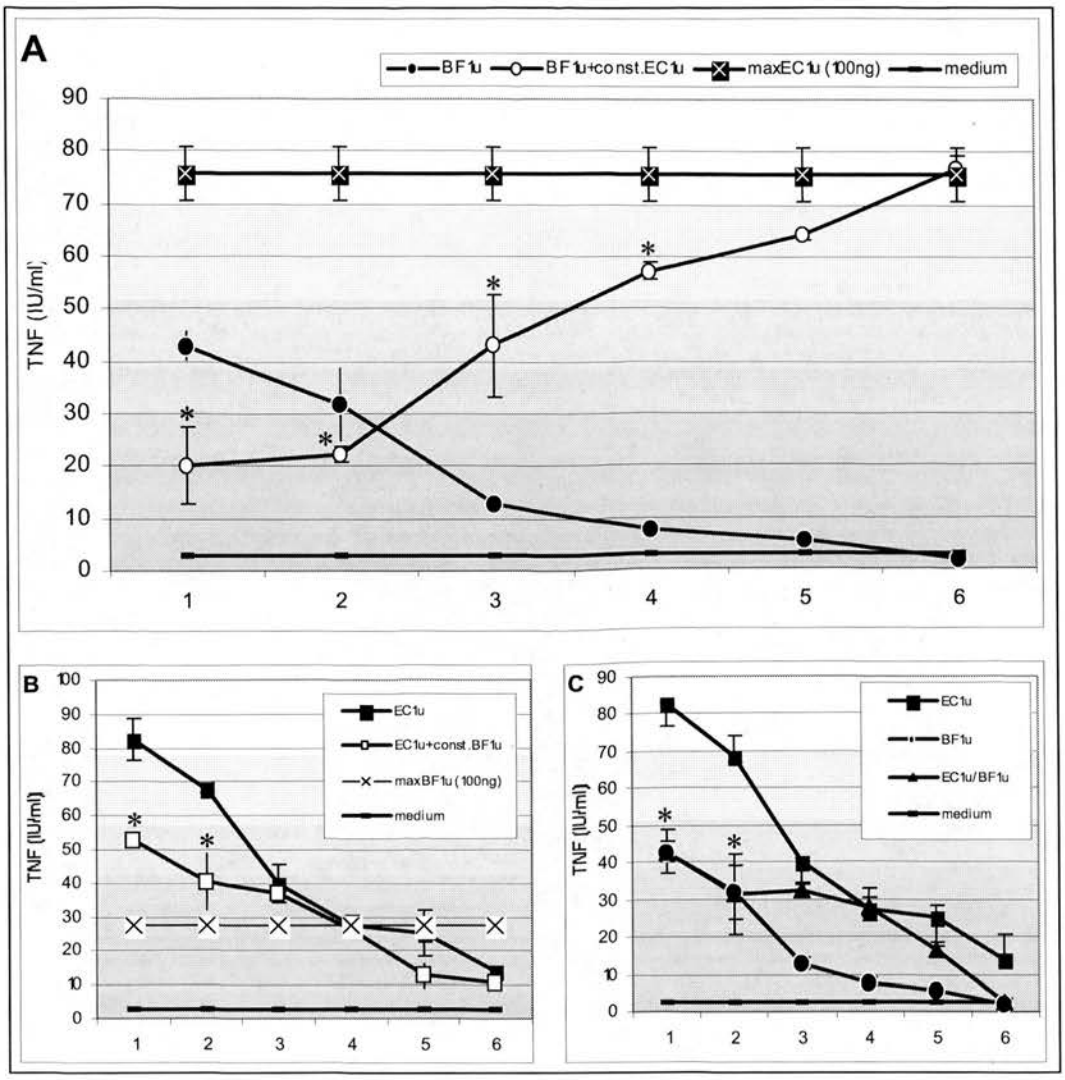

Figure 3.23 TNFα production (IU/ml) by THP-1 cell with VD3 treatment after interactions between EC1u and BF1u in different combinations and measured by ELISA

(A) Comparing between various concentrations of BF1u LPS alone and various concentrations of BF1u LPS plus constant concentration of EC1u (100ng/ml). (B) Comparing between various concentrations of EC1u LPS alone and various concentration of EC1u LPS plus constant concentration of BF1u (100ng/ml). (C) Comparing between various concentration of EC1u LPS alone, various concentrations of BF1u LPS alone and various mixtures of equal concentrations of EC1u and BF1u. Results represent the means +/- SEM for at least three experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

1= 1000ng/ml, 2=100ng/ml, 3=10ng/ml, 4=1ng/ml, 5=0.1ng/ml, 6=0.01ng/ml.

Fig 3.23A demonstrates a substantial lowering effect that goes even below the values for the two highest BF1u LPS concentrations and continues in a dose-dependent style to the lowest concentration of BF1u. This effect is statistically significant at the first four concentrations. A slight fluctuating lowering effect is demonstrated in Fig 3.23B and it is significant at 1000 and 100ngs. Fig3.23C also shows a significant differences between EC1u/BF1u and EC1u at 1000 and 100ngs ($p<0.05$).

3.6 Interactions between different LPSs from various bacterial species in stimulation IL-β production

3.6.1 Interactions between EC1u and BF1u LPSs on IL-β production by human PBMC and measured by ELISA

Using an ELISA to measure IL-β production after challenging human PBMC, an interaction investigation was done between EC1u and BF1u LPSs in the manner identical to that shown in Section 3.4.1. Fig 3.24A shows a slight lowering effect of BF1u LPS against a constant concentration of 100ng/ml of EC1u in most concentrations by a range of ten to twenty IL-β units compared to the constant EC1u response. The effect is significant at the two highest concentrations of BF1u. Fig 3.24B shows also a slight lowering effect exerted by constant BF1u and becomes significant at the lower concentrations of 10, 1, 0.1ngs. Fig 3.24C demonstrated no significant differences between different concentrations except between EC1u/BF1u and BF1u at the first three highest concentrations ($p<0.05$).

3.6.2 Interactions between EC2u and BF2u LPSs on IL-β production by human PBMC and measured by ELISA

Fig 3.25A demonstrates a non-significant lowering effect of BF2u LPS on the constant amount of EC2u LPS . The highest lowering effects are at 1000ng/ml and 1ng/ml of BF2u LPS. Although it is non-significant, Fig 3.25B shows an obvious lowering effect of 100ng/ml BF2u against EC2u LPS stimulated IL-β production at concentrations of 100, 10, 1, 0.1 ng/ml of EC2u LPS.

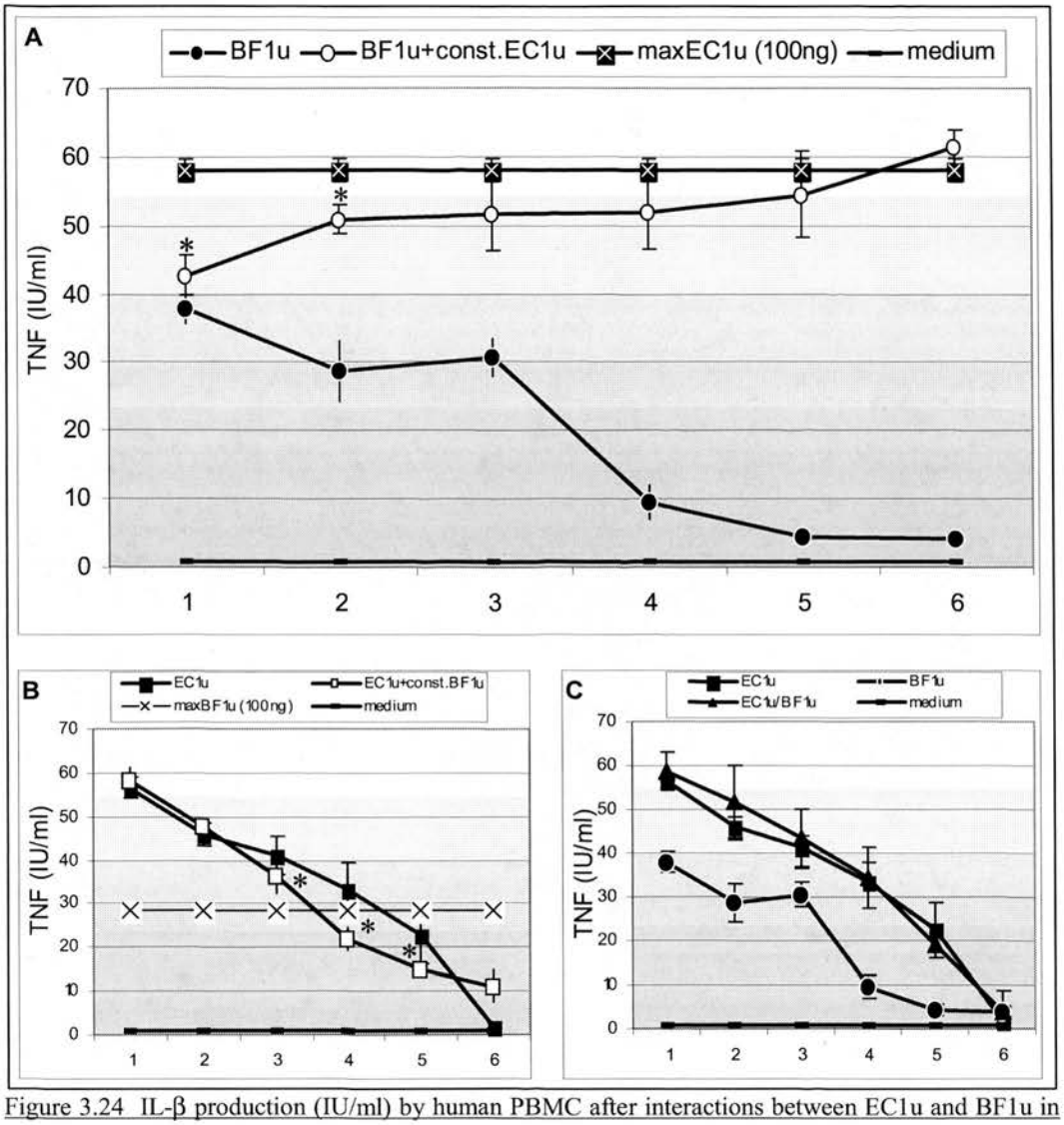

Figure 3.24 IL-β production (IU/ml) by human PBMC after interactions between EC1u and BF1u in different combinations and measured by ELISA

(A) Comparing between different concentrations of BF1u LPS alone and different concentrations of BF1u LPS plus constant concentration of EC1u (100ng/ml). maximum of EC1u concentration alone at 100ng/ml. (B) Comparing between different concentrations of EC1u LPS alone and different concentration of EC1u LPS plus constant concentration of BF1u (100ng/ml). Maximum of BF1u concentration alone at 100ng/ml. (C) Comparing between different concentration of EC1u LPS alone, different concentrations of BF1u LPS alone and different concentration of equal concentrations of mixed EC1u and BF1u. Results represent the means +/- SEM for at least three experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

1= 1000ng/ml, 2=100ng/ml, 3=10ng/ml, 4=1ng/ml, 5=0.1ng/ml, 6=0.01ng/ml.

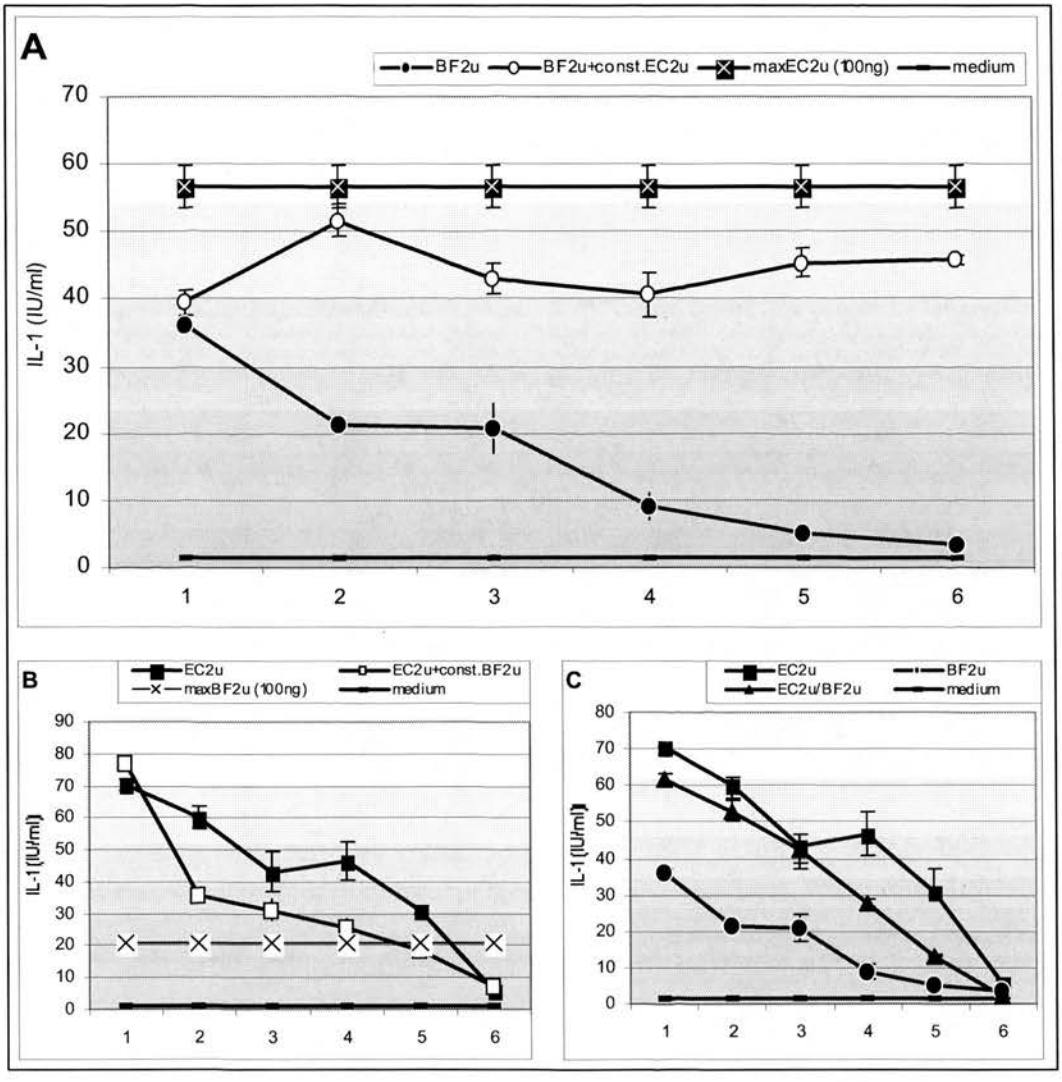

Figure 3.25 IL-β production (IU/ml) by human PBMC after interactions between EC2u and BF2u in different combinations and measured by ELISA

(A) Comparing between different concentrations of BF2u LPS alone and different concentrations of BF2u LPS plus constant concentration of EC2u (100ng/ml). Maximum of EC2u concentration alone at 100ng/ml. (B) Comparing between different concentrations of EC2u LPS alone and different concentration of EC2u LPS plus constant concentration of BF2u (100ng/ml). Maximum of BF2u concentration alone at 100ng/ml. (C) Comparing between different concentration of EC2u LPS alone, different concentrations of BF2u LPS alone and different concentration of equal concentrations of mixed EC2u and BF2u. Results represent the means +/- SEM for at least three experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

1= 1000ng/ml, 2=100ng/ml, 3=10ng/ml, 4=1ng/ml, 5=0.1ng/ml, 6=0.01ng/ml.

3.6.3 Interactions between EC3u and BF3u LPSs on IL-β production by human PBMC and measured by ELISA

Fig 3.26A shows an almost non-significant constant lowering effect of BF3u LPS against IL-β production by a constant EC3u LPS. Moreover, Fig 3.26B does not demonstrate a significant lowering effect of the constant BF3u against EC3u LPS.

3.6.4 Interactions between EC4u and BF4u LPSs on IL-β production by human PBMC and measured by ELISA

Fig 3.27A indicates an obvious example of fluctuating lowering effect which reach the most and significant effect at 100 and 0.1 ng/ml of BF4u. A slight non-significant lowering effect is also noticeable in Fig 3.27B starting from concentration of 100ng/ml of EC4u LPS. An additive significant effect is seen when EC4u and BF4u LPS is added together in equal amounts (Fig 3.27C) at 1000, 100, 10 and 1ngs concentrations ($p<0.05$).

3.6.5 Interactions between EC1u and BF1u LPSs on IL-β production by THP-1 cells and measured by ELISA

Using ELISA assay to measure the IL-β production after challenging THP-1 cells, an interaction approaches were done between EC1u and BF1u LPSs in the manner identical to that shown in Section 3.4.1. Fig 3.28A shows the lowering effect of the different BF1u against constant concentration of 100ng/ml of EC1u in dose-dependent style and it is statistically significant just for the two higher concentrations of BF1u. This effect is totally reduced starting from 10ng/ml of BF1u. Significant lowering effect is observed in Fig 3.29A at concentrations of 10 and 1ng/ml of BF1u. Fig 3.28B shows a slight significant lowering effect at 1ng/ml of EC1u. While Fig 3.29B shows non-significant differences between EC1u+constBF1u and EC1u. EC1u/BF1u shows significant differences against EC1u at some lower concentrations as shown in both Fig 3.28C and Fig3.29C ($p<0.05$).

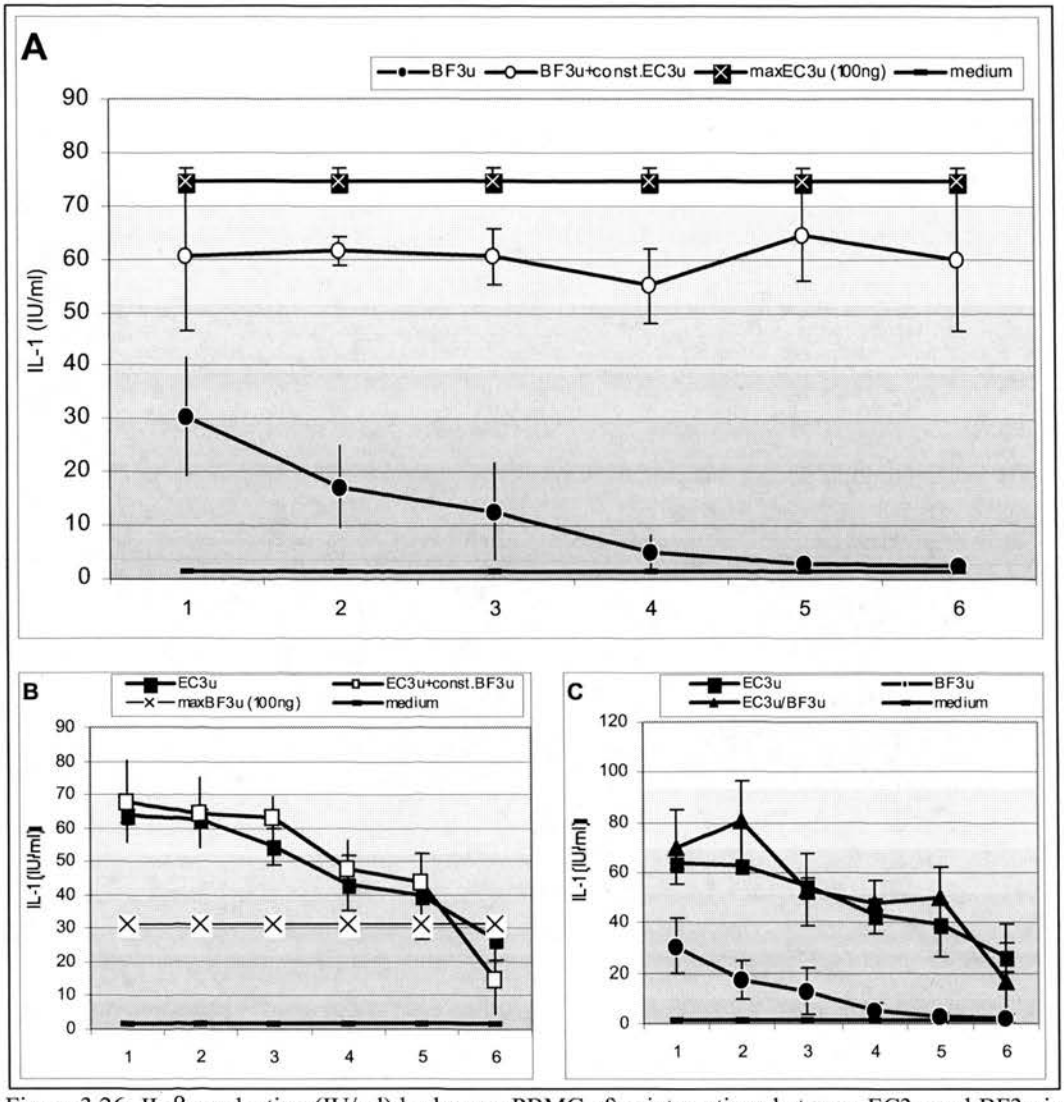

Figure 3.26 IL-β production (IU/ml) by human PBMC after interactions between EC3u and BF3u in different combinations and measured by ELISA

(A) Comparing between different concentrations of BF3u LPS alone and different concentrations of BF3u LPS plus constant concentration of EC3u (100ng/ml). Maximum of EC3u concentration alone at 100ng/ml. (B) Comparing between different concentrations of EC3u LPS alone and different concentration of EC3u LPS plus constant concentration of BF3u (100ng/ml). Maximum of BF3u concentration alone at 100ng/ml. (C) Comparing between different concentration of EC3u LPS alone, different concentrations of BF3u LPS alone and different concentration of equal concentrations of mixed EC3u and BF3u. Results represent the means +/- SEM for at least three experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

1= 1000ng/ml, 2=100ng/ml, 3=10ng/ml, 4=1ng/ml, 5=0.1ng/ml, 6=0.01ng/ml.

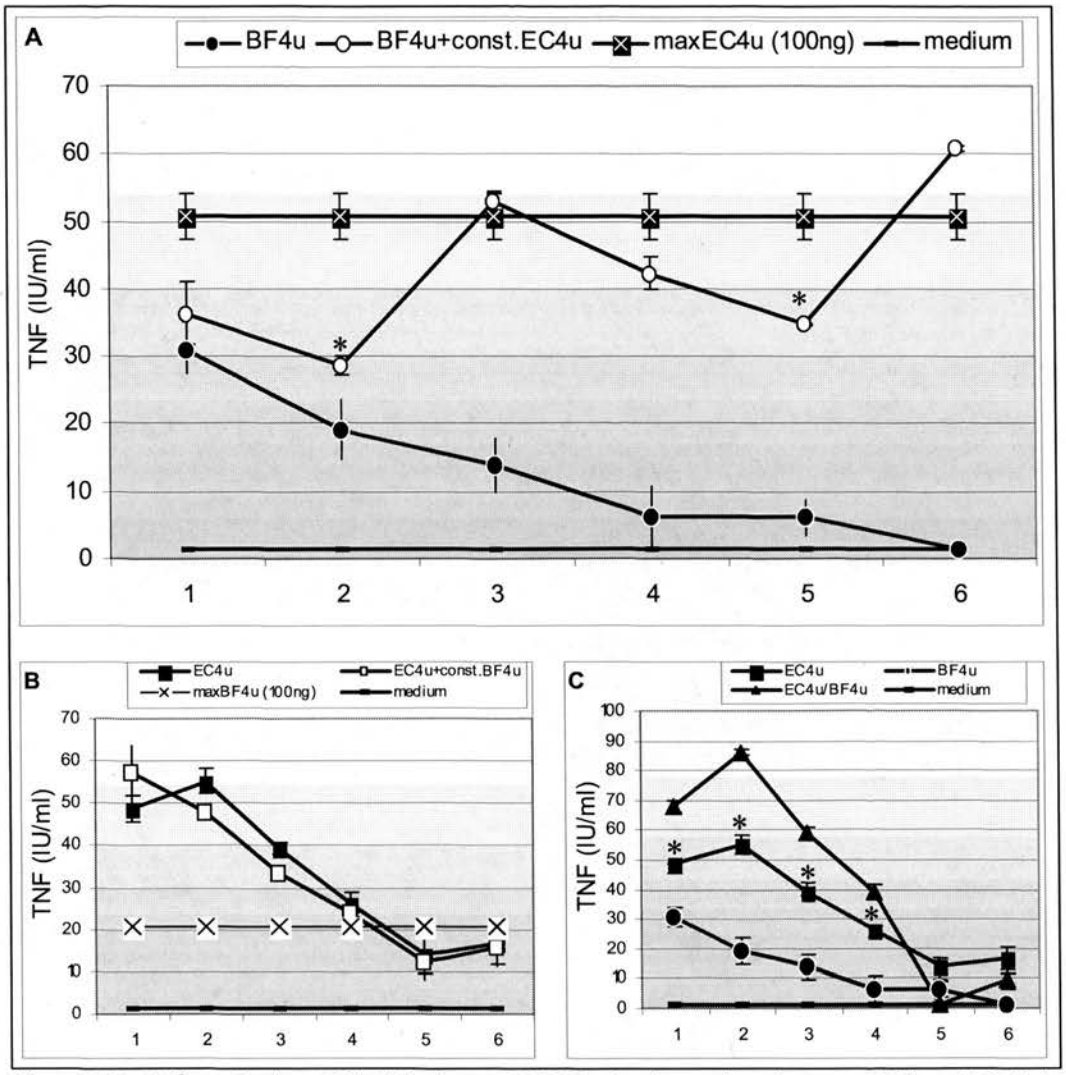

Figure 3.27 IL-β production (IU/ml) by human PBMC after interactions between EC4u and BF4u in different combinations and measured by ELISA

(A) Comparing between different concentrations of BF4u LPS alone and different concentrations of BF4u LPS plus constant concentration of EC4u (100ng/ml). maximum of EC4u concentration alone at 100ng/ml. (B) Comparing between different concentrations of EC4u LPS alone and different concentration of EC4u LPS plus constant concentration of BF4u (100ng/ml). maximum of BF4u concentration alone at 100ng/ml. (C) Comparing between different concentration of EC4u LPS alone, different concentrations of BF4u LPS alone and different concentration of equal concentrations of mixed EC4u and BF4u. Results represent the means +/- SEM for at least three experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

1= 1000ng/ml, 2=100ng/ml, 3=10ng/ml, 4=1ng/ml, 5=0.1ng/ml, 6=0.01ng/ml.

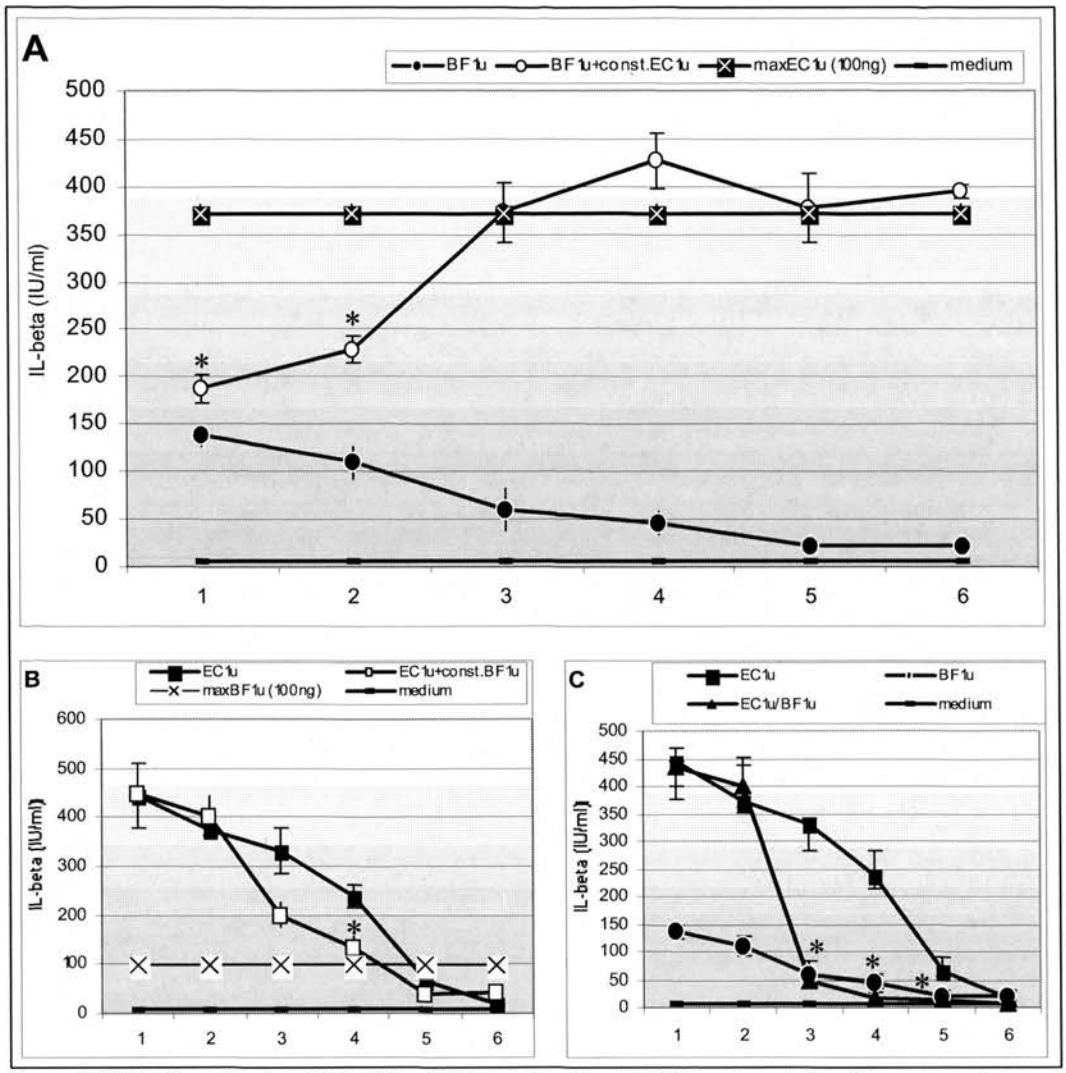

Figure 3.28 IL-β production (IU/ml) by THP-1 cell without VD3 treatment after interactions between EC1u and BF1u in different combinations and measured by ELISA

(A) Comparing between different concentrations of BF1u LPS alone and different concentrations of BF1u LPS plus constant concentration of EC1u (100ng/ml). maximum of EC1u concentration alone at 100ng/ml. (B) Comparing between different concentrations of EC1u LPS alone and different concentration of EC1u LPS plus constant concentration of BF1u (100ng/ml). Maximum of BF1u concentration alone at 100ng/ml. (C) Comparing between different concentration of EC1u LPS alone, different concentrations of BF1u LPS alone and different concentration of equal concentrations of mixed EC1u and BF1u. Results represent the means +/- SEM for at least three experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

1= 1000ng/ml, 2=100ng/ml, 3=10ng/ml, 4=1ng/ml, 5=0.1ng/ml, 6=0.01ng/ml.

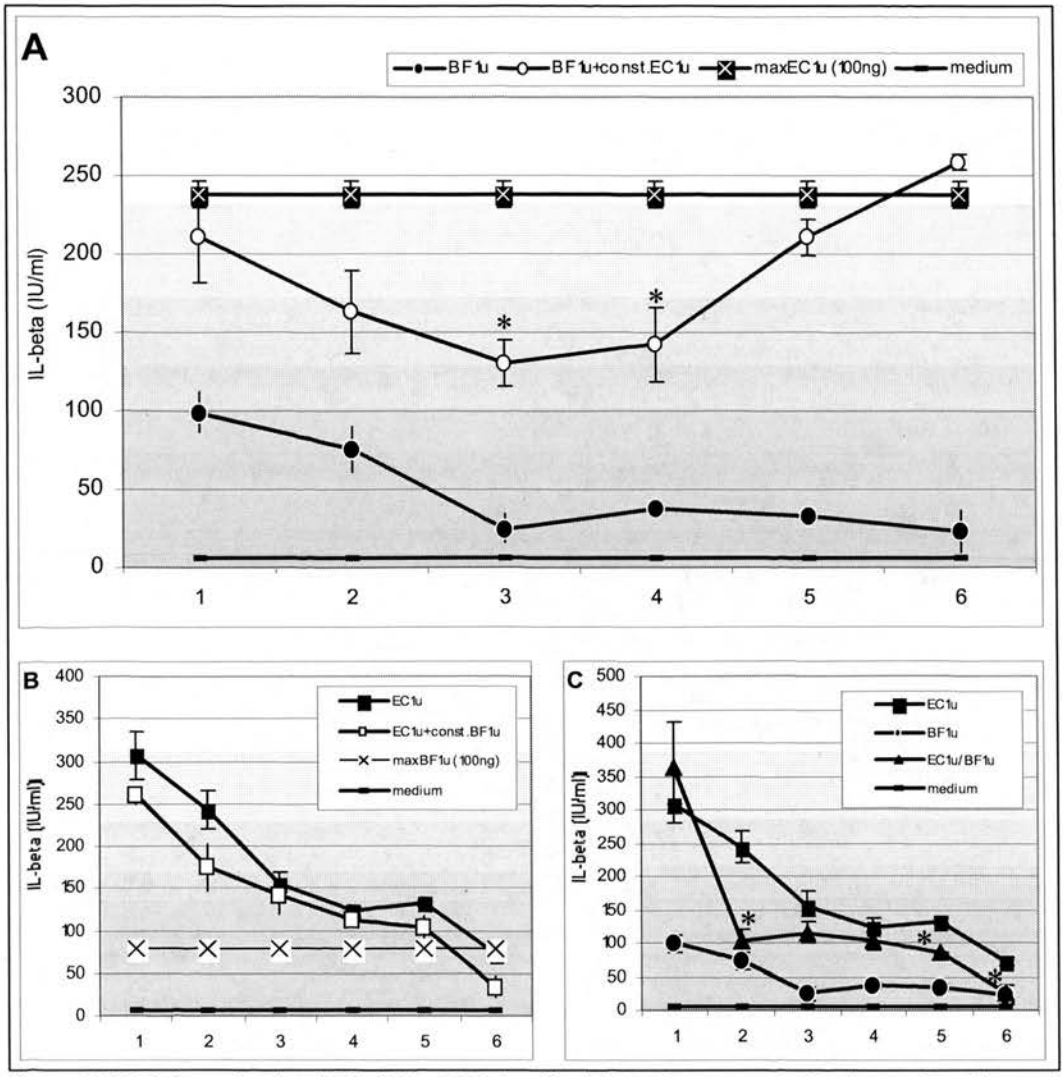

Figure 3.29 IL-β production (IU/ml) by THP-1 cell with VD3 treatment after interactions between EC1u and BF1u in different combinations and measured by ELISA

(A) Comparing between different concentrations of BF1u LPS alone and different concentrations of BF1u LPS plus constant concentration of EC1u (100ng/ml). maximum of EC1u concentration alone at 100ng/ml. (B) Comparing between different concentrations of EC1u LPS alone and different concentration of EC1u LPS plus constant concentration of BF1u (100ng/ml). Maximum of BF1u concentration alone at 100ng/ml. (C) Comparing between different concentration of EC1u LPS alone, different concentrations of BF1u LPS alone and different concentration of equal concentrations of mixed EC1u and BF1u. Results represent the means +/- SEM for at least three experiments. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

1= 1000ng/ml, 2=100ng/ml, 3=10ng/ml, 4=1ng/ml, 5=0.1ng/ml, 6=0.01ng/ml.

3.7 Toll like receptor assay

Using a transient transfection of TL4/MD2, TLR2, CD14 receptors into HEK-293 cells, comparison experiments were done for five unpurified and five purified *B. fragilis* LPS preparations extracted by different methods in addition to unpurified and purified *R. sphaeroides* LPS. Heat killed *B. fragilis* from three different populations of different polysaccharide capsules were also examined for toll like receptor specificity. These experiments were done with and without transfection efficiency control.

3.7.1 Toll like receptor specificity of unpurified *B. fragilis* LPSs

Fig 3.30A and B clearly demonstrate the TLR2 high specificity of all five unpurified *B. fragilis* LPS preparations. All of them show stronger TLR2 specificity than the TLR2 positive control LPS except BF3u which show an equal TLR2 specificity to that of the TLR2 positive control LPS. BF1u LPS shows the strongest TLR2 specificity among all others. Non of the five unpurified *B. fragilis* LPS preparations demonstrate a TLR4 specificity comparing with the TLR4 positive control LPS. No considerable differences between running a transfection assay with or without the transfection efficiency control were observed. All TLR2 specificity of five unpurified *B. fragilis* LPS preparations show a significant difference compared to the values of TLR4/MD2 and CD14 with or without using transfection efficiency assay. BF1u shows significant difference compared to BF3u when the transfection efficiency assay was done (Fig 3.30B).

3.7.2 Toll like receptor specificity of purified *B. fragilis* LPSs

Fig 3.31A and B clearly demonstrate the TLR2 high specificity of BF1p, BF4p and BF5p LPSs and reasonable TLR2 specificity with BF2p and BF3p which were extracted by TMP and PCP respectively. The specificity of TLR2 positive control LPS is stronger than all five purified *B. fragilis* LPS preparations. BF5p LPS shows the strongest TLR2 specificity among all others followed by BF1p and BF4p.

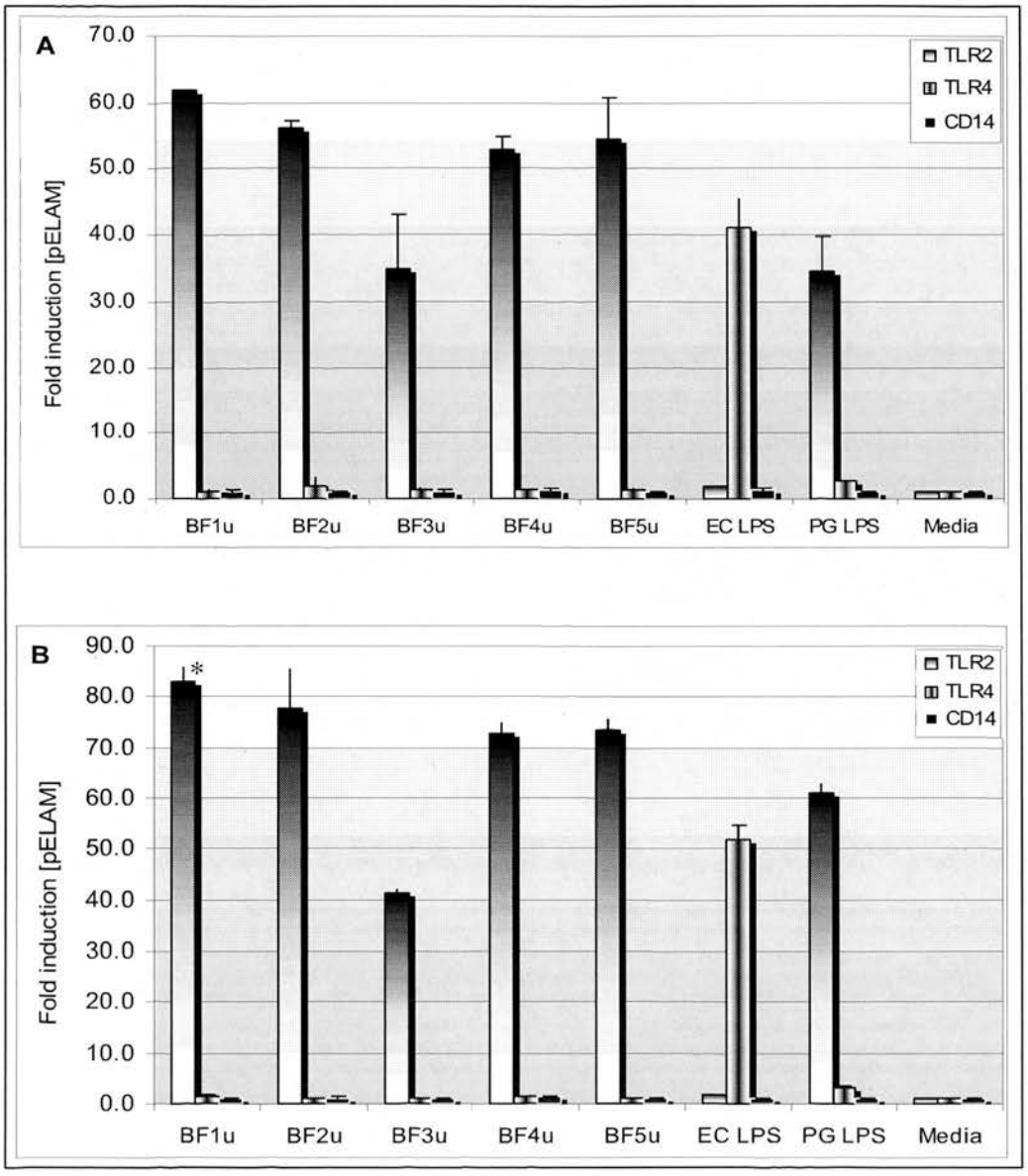

Figure 3.30 Toll like receptor specificity of five unpurified *B. fragilis* LPS preparations

(A) HEK-293 transfection assay without transfection efficiency control (B) HEK-293 transfection assay with transfection efficiency control. (EC LPS) TLR4/MD2 positive control of *E. coli* LPS (PG LPS) TLR2 positive control of *P. gingivalis* LPS. Results represent the means +/- SEM for three experiments including the transfection efficiency control experiment. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

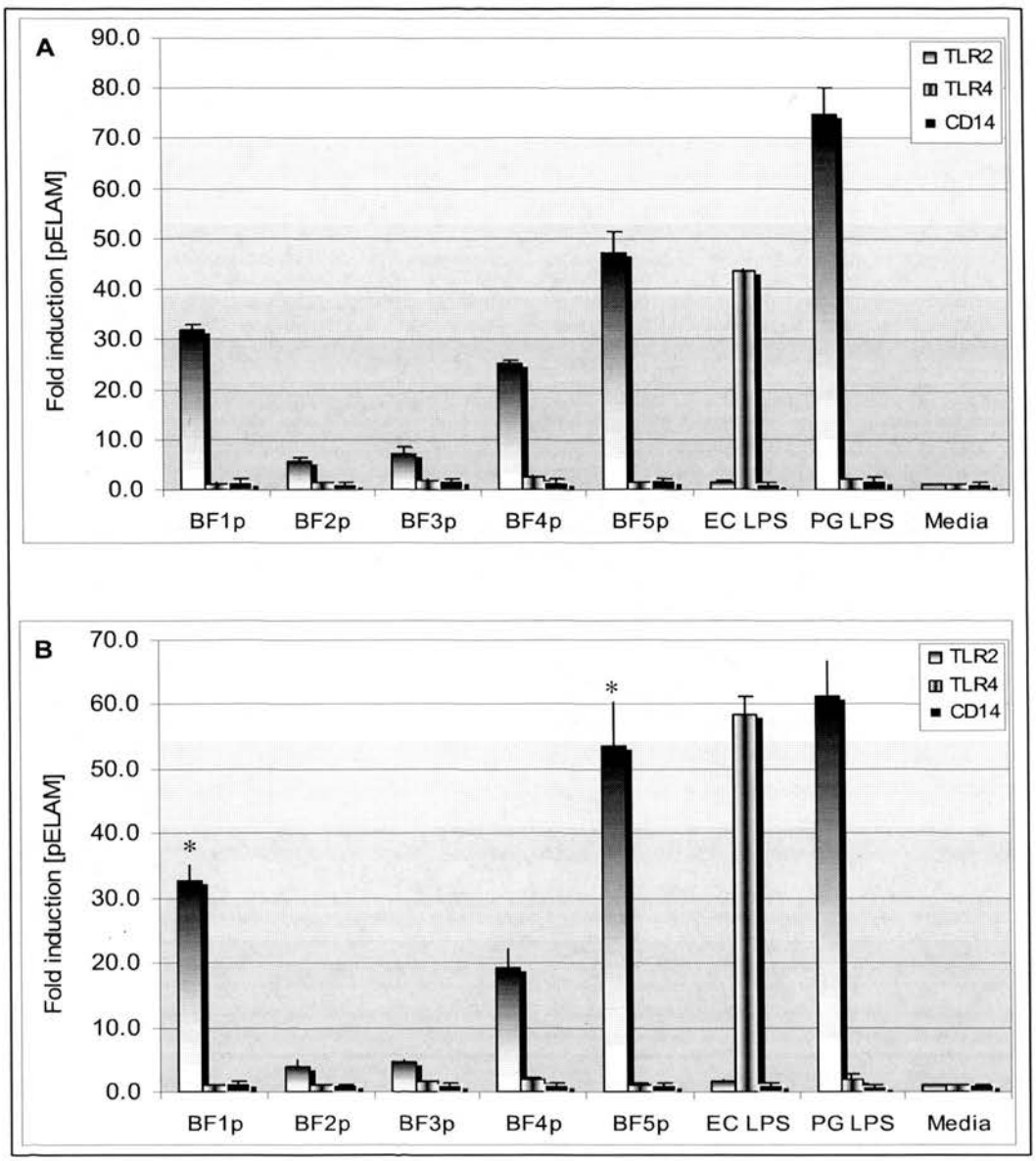

Figure 3.31 Toll like receptor specificity of five purified *B. fragilis* LPS preparations

(A) HEK-293 transfection assay without transfection efficiency control (B) HEK-293 transfection assay with transfection efficiency control. (EC LPS) TLR4/MD2 positive control of *E. coli* LPS (PG LPS) TLR2 positive control of *P. gingivalis* LPS. Results represent the means +/- SEM for three experiments including the transfection efficiency control experiment. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

None of the five purified *B. fragilis* LPS preparations demonstrate a TLR4 specificity compared with the TLR4 positive control LPS. All TLR2 specificity of five purified *B. fragilis* LPS preparations show a significant difference compared to the values of TLR4/MD2 and CD14 only with using the transfection efficiency assay. BF1p and BF5p show significant difference compared to BF2p and BF3p when the transfection efficiency assay was done (Fig 3.31B).

3.7.3 Toll like receptor specificity of heat killed *B. fragilis* and *R. sphaeroides* LPSs

Fig 3.32A and B clearly demonstrate the TLR2 high specificity of HK EDL, HK SC and HK LC heat killed *B. fragilis* populations. All of these heat killed populations are stronger than TLR2 positive control LPS in their TLR2 specificities. In the same time, none of them demonstrate TLR4 specificity comparing with the TLR4/MD2 positive control LPS. *R. sphaeroides* LPSs show weak TLR2 specificity in transfection assay without efficiency control and show much weaker TLR2 specificity in transfection assay with efficiency control. Nevertheless all TLR2 specificity of three heat killed *B. fragilis* populations and two samples of *R. sphaeroides* LPSs show a significant differences compared to the values of TLR4/MD2 and CD14 only with using transfection efficiency assay.

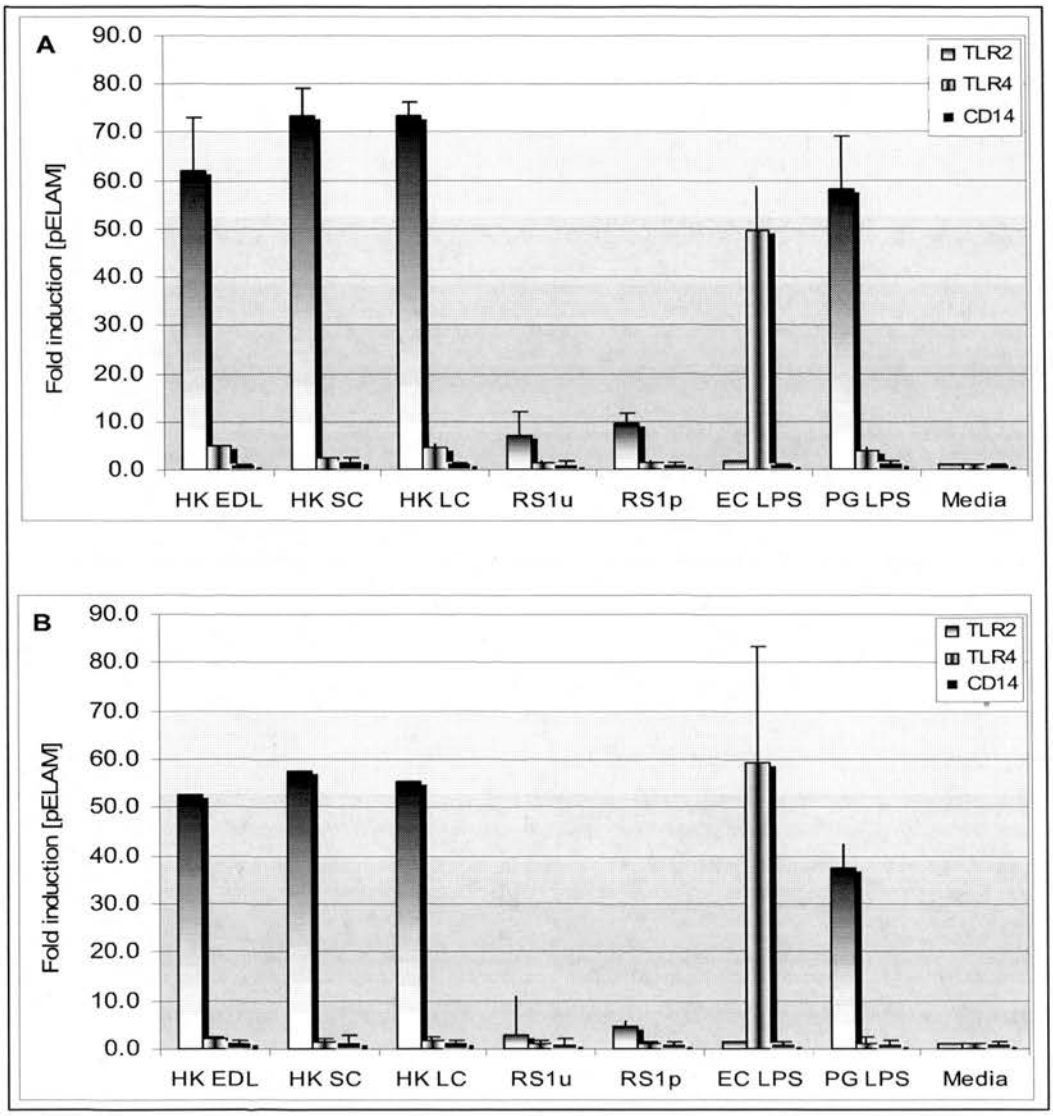

Figure 3.32 Toll like receptor specificity of heat killed *B. fragilis* and *R. sphaeroides* LPSs

(A) HEK-293 transfection assay without transfection efficiency control (B) HEK-293 transfection assay with transfection efficiency control. (EC LPS) TLR4/MD2 positive control of *E. coli* LPS (PG LPS) TLR2 positive control of *P. gingivalis* LPS (HK EDL) heat killed *B. fragilis* of electron density layer (HK SC) heat killed *B. fragilis* of small capsule (HK LC) heat killed *B. fragilis* of large capsule (RS1u) unpurified *R. sphaeroides* LPS (RS1p) purified *R. sphaeroides* LPS. Results represent the means +/- SEM for three experiments including the transfection efficiency control experiment. * Indicates data point represent a statistical difference from other data points ($p<0.05$).

CHAPTER 4: DISCUSSION

4.1 Lipopolysaccharide extraction and analysis

The actual dialogue which is taking place between the innate immune mechanisms and different PAMP molecules is far from being completely elucidated especially when it is comes to Gram-negative bacteria. Although it is crucial to identify the detailed interactions between the focal inflammatory inducer, endotoxin and the innate immune mechanisms, it is not possible to isolate these interactions from possible interactions with other cell wall related PAMPs such as capsular polysaccharide, lipoprotein and peptidoglycan. In addition, it is fundamental to try to mimic the bacterial behaviour inside the host as much as possible to make the investigation approach more reliable. Studies have used different extraction and purification methods which make comparisons difficult. These different techniques affect the purity, solubility and efficacy of the LPS preparations that are to be worked with. In this context, it is observed that LPS activity can differ significantly according to the type of organism, method of cultivation and the technique by which the LPS is extracted (Fukushi *et al.*, 1964; Morrison and Leive, 1975). Moreover, although LPS activity is particularly associated with the Lipid A moiety it can also be affected by other materials which can be present in the final preparation such as outer membrane proteins (Morrison *et al.*, 1976), the specific O-antigen subunit (Vukajlovich and Morrison, 1985) and the O-antigen carbohydrates (Morrison *et al.*, 1987). The associated material is not always of protein in nature. It is assumed that the contaminants of *B. fragilis* LPS are not of protein origin (Mancuso *et al.*, 2005). A previous study found that there are non-protein components present in enterobacterial preparation that have LPS-like activities (Muroi *et al.*, 2003). Moreover, one early study using purified capsular polysaccharide of *B. fragilis* showed that these molecules have the capacity to induce IL-8 production from human monocytes or polymorphonuclear leukocytes (Gibson *et al.*, 1996). Furthermore, LPS in itself is a particularly difficult ligand to work with because of its amphipathicity which neither make it easily soluble nor allow the forming of LPS monomers in aqueous suspension (Lien *et al.*, 2000).

In this study four different extraction methods were chosen to produce different forms of LPS; mainly smooth, high molecular weight material is extracted by aqueous phenol (AP) method. With Phenol, Chloroform, Petroleum spirit (PCP)

method, rough, low molecular weight material predominates. Using the last two extraction methods: Triton/magnesium chloride with and without Protienase K treatment(TM/TMP) method and Boiling water with Proteinase K treatment (BWP) method which are much milder than the first two methods as proposed by Poxton and Edmond (1995). LPS yields from all methods with all tested bacteria, except of *R. sphaeroides*, were reasonable. For example, the percentage of LPS yield, extracted by AP method from *E. coli* was 1.2% of the bacterial cell dry weight (Table 3.1) which is in agreement with previous studies that reported the percentage of LPS yield extracted by the same method from the same organism to be from 1 to 4% (Luderitz *et al.*, 1971) However, the yield and solubility of *B. fragilis* LPS extracted by PCP were much lower compared to others. It is reported that applying the PCP method to extract LPS from *B. fragilis* dry bacteria results in only a trace amount of the LPS (Kasper, 1976; Hofstad *et al.*, 1977). This possibly happens because of the effect of the capsular polysaccharide which makes the cell envelope more hydrophilic to the extent it becomes insoluble in the hydrophobic PCP mixture (Weintraub *et al.*, 1985). It is also generally recognised that the solubility of rough LPS in water is poor (Hellman *et al.*, 2003). If it comes to choosing a common method that has an ability to extract smooth and rough LPS in the same time, then TM/TMP will be the preferable choice since in addition to its mild nature, the LPS preparations extracted by this method showed a high level of purity especially after applying proteinase K treatment. The AP method is considered to be the most common LPS extraction method (Yi and Hackett, 2000) and showed high level of cleanness with LPS extracted from *E. coli* (Fig 3.1A). This was also documented with this extraction method which usually results in a pure LPS preparation of the smooth type (Darveau and Hancock, 1983). Furthermore, although PCP method was documented to be more effective in extraction of rough type LPS than the AP method (Darveau and Hancock, 1983), it does not seem to produce a cleanness of appearance when it was applied to *B. fragilis* (BF3u in Fig 3.2) compared with the appearance of the LPS preparation of the same organism extracted by TM/TMP or AP methods (BF1u, BF2u and BF5u in Fig 3.2).

It is widely believed that highly purified LPS is essential for studying its biological activity in vitro (Kutuzova *et al.*, 2001). Although it is not an easy task to approach

since it was early demonstrated (1960s) that during extraction, when LPS is subjected to denaturing steps many of the contaminating protein and other substances become tightly bound to the LPS molecule and cannot be detached completely (Rudbach and Proctor, 2001). So, use of a repurification procedure to prepare LPS preparation in highly purified form has been one of the important goals in the LPS research field especially when it is intended to investigate the specific signalling activity of the LPS (i.e. the TLR specificity). For example, procedures of using gel filtration (Gu and Tsai, 1991), repeating ultra centrifugation step (Yi and Hackett, 2000), digestion with proteinase-K (Chart and Rowe, 1991), using silica cartridge (Qureshi *et al.*, 1991) or even repeating the extraction procedure (Perez Perez and Blaser, 1985; Yi and Hackett, 2000) are used to achieve highly purified LPS. The repurification method of Manthey and Vogel (1994) which was used in this study was tried by many investigators until Hirschfeld and his colleagues (2000) gave this method another dimension since it was used to verify the actual TLR specificity of LPS through eliminating the protein contaminants which are usually associated with LPS preparations. Application of this repurification method has a noticeable effect on the appearance of some LPS preparations tested in this study which is obvious when comparing samples like BF3u and BF3p which were extracted by PCP method, BF4u and BF4p which were extracted by BWP method, EC2u and EC2p which were extracted by TM method and EC4u and EC4p which were extracted by BWP method (Fig 3.1 and 3.2). Controversially, this seem to favour using lower concentration of proteinase K treatment in the TMP method but against the use of higher concentration of proteinase K treatment in BWP method when it is purposed to eliminate the protein contaminants from LPS preparation by using proteinase K treatment. This was illustrated further by the comparison between the appearance of EC1u and EC2p which were extracted by the TMP method as showed in Fig 3.1C. Moreover, the effect of using the repurification method was very much noticeable when BF1u, BF2u, BF3u, BF4u and BF5u were compared with BF1p, BF2p, BF3p, BF4p and BF5p in term of their intensity of TLR2 specificity as demonstrated by Fig 3.30 and Fig 3.31. On the other hand, it is totally unreasonable to consider that the non-purified LPS is without its uses since it is still a powerful tool for studying an immune inflammatory response against LPS. After all these non-purified LPS

preparations more closely mimic the LPS that would be encountered by host cells infected with either whole bacteria or released LPS molecules. For the same reason, different heat killed bacteria populations were involved in the TLR assay (discussed later). In fact one study supports this notion since it reported that fluorescein labelled *E. coli* was able to pass across the rat intestinal epithelium but fluorescein conjugated *E. coli* LPS was unable to do so even after disruption of the intestinal epithelium with a potent mucolytic agent which did not result in significant increase in transmucosal passage of *E. coli* LPS (Benoit *et al.*, 1998). The ability to penetrating the intestinal barrier when it is happen, expreses LPS directly to immune cells such as monocytes which were used in this study. As a conclusion, using both LPS extraction methods and an extra technique to repurify LPS contributed much to this study in terms of comparing different LPS preparations of different levels of purity.

SDS-PAGE analysis is often used to differentiate between rough and smooth types of LPS. However, this method of LPS analysis does not identify every immunoreactive material (Fomsgaard *et al.*, 1990). The smooth LPS with a ladder-like pattern on SDS-PAGE analysis of both *E. coli* and *Ps. aeruginosa* (Fig3.1 and Fig3.3) is something widely recognised in the literature. As a rule, smooth LPS shows a high degree of heterogenicity and demonstrates LPS molecules with different numbers of repeating oligosaccharide units (Fomsgaard *et al.*, 1990). SDS-PAGE analysis of *B. fragilis* LPS usually consists of two deeply stained bands constituting the core regions. One study reported that most *B. fragilis* strains have a rough LPS style which runs at the gel front followed by a common antigen and a ladder pattern bands of smooth LPS (Poxton and Brown, 1986). Other investigators did not demonstrate such a smooth LPS in any of the 17 strains examined including NCTC 9343 strain (Weintraub *et al.*, 1985). The findings of this study did not show an obvious ladder pattern in any of *B. fragilis* LPSs. Nevertheless, PCP and to lesser extent BWP methods produced an intense LPS material along the gel which seems to be kind of a strong ladder pattern as demonstrated in Fig3.2A. Moreover, the high molecular weight material observed distinctively in this study with *B. fragilis* LPS preparations that were extracted by TM and TMP methods (Fig3.2A, BF1u & BF2u) are similar to that found by another study of the same strain, *B. fragilis* NCTC 9343 (Poxton and Edmond, 1995). It was also reported in the same study that the proteinase K

treatment itself was not responsible for any biological activity (Poxton and Edmond, 1995).

An overall view of the protein content of the LPS preparations which were analysed by colloidal gold stain showed that BWP extraction method is effective in eliminating protein even before applying the repurification method as demonstrated by Fig3.1B, EC4u; Fig3.2B, BF4u and Fig3.3B, PA4u. This may be because this method includes a treatment with proteinase K at relatively high concentration. The TMP method showed a similar effective protein elimination before applying repurification with *B. fragilis* only (Fig3.2B, BF2u). It has been reported that the sensitive colloidal gold stain can detect as little as 1ng of protein so if no bands appeared in any of the LPS preparation tested it means that the protein contaminants is always under 0.02% (Mancuso *et al.*, 2005).

Another approach to analyse the LPS content was done by applying the LAL assay. This analysis is widely recognised as an official test to substitute for the rabbit pyrogen test. The LAL assay is one of the most sensitive and highly specific methods for detecting the existence of endotoxin and comparing the endotoxin intensity between different LPS preparations (Nakagawa *et al.*, 2002). However, it is not known if LPS activity in this assay is linked to the ability of LPS to induce inflammatory immune responses for reasons like the obvious phylogenic distance between the horseshoe crab and human cells (Nakagawa *et al.*, 2002). In fact, one previous study reported that there is not a strong relationship between LAL activity of specific LPS and its in vitro TNF-α induction effect (Luchi and Morrison, 2000).

4.2 Production and detection of TNF-α and IL-1β cytokines

Two different cell types were used to study cytokines induced by LPS: human monocytes isolated from healthy volunteers and the THP-1 cell line. Using fresh blood sample is the most reliable way to mimic what happens inside the host, while using the THP-1 cell line overcomes donor variations and availability. Although cell lines of transformed origin have an ease of use advantage over the human macrophages which although are easily cultured in vitro they have intrinsic limitations in term of numbers, lifetime and heterogeneity in their responses between

different donors (Witsell and Schook, 1991; Ravasi *et al.*, 2002; Perkins and Gilmore, 2006). However, it was reported that there are some differences in NF-κB signalling between transformed and primary cells (Smith *et al.*, 2001). Regarding the use of THP-1 cells in particular, it has been reported in a recent article that LPS stimulates an identical transcriptional response in both the THP-1 cell line and human peripheral blood mononuclear cells (Sharif *et al.*, 2007). This finding makes the THP-1 cell line one of a preferred model system for studying the mechanisms of LPS and NF-κB dependent gene expression (Sharif *et al.*, 2007).

The result of cytokine production using THP-1 cell line are demonstrated in Fig 3.14 - 3.23, 3.28 and 3.29. These figures showed that untreated THP-1 cells produce more cytokine than Vitamin D3-treated THP-1 cells which is unexpected since Vitamin D3 treatment is supposed to induce untreated THP-1 cells to mature into monocyte-like cells. The reason for this discrepancy between the result of this study and result of another study like that of Delahooke and others (1995) in the induction of THP-1 by Vitamin D3 to be mature monocytes is unclear, but it may result from growth conditions or cell passage number. For example the THP-1 cells used in this study were from totally new THP-1 stock and not from an old passage. It is recognised that Vitamin D3 is effective in stimulating the expression of CD14 in THP-1 cells in particular as one study showed that Vitamin D3-treated THP-1 cells expressed up to 380,000 molecules CD14/cell compared to levels of 50 to 100,000 CD14 molecules/monocyte and around 10,000 CD14 molecules/cell on untreated THP-1 cells (Tobias *et al.*, 1993). Nevertheless, another study indicated that the expression of CD14 is not fundamentally important. It was shown that although after adding Vitamin D3, the level of CD14 was increased slightly in THP-1 cells and strongly in another kind of cell, Mono Mac 6 cells, the TNF-α response was much stronger in THP-1 cells compared with Mono Mac 6 cells (Landmann *et al.*, 1998). In the same context, a previous study found that the untreated THP-1 cells expressed MD-2 and TLR4 without any stimulation with molecules such as Vitamin D3 (Shimazu *et al.*, 1999b). Another study demonstrated that THP-1 cells endogenously express TLR2 (Kirschning *et al.*, 1998). A third study showed that untreated THP-1 cells expressed twice the level of TLR2 compared to Vitamin D3-treated THP-1 cells (Yang *et al.*, 2001). The same study also showed that there is no real differences in the TLR4

expression between VD3-treated and untreated THP-1 cells (Yang *et al.*, 2001). Moreover, a recent study has also demonstrated that VD3 suppresses the expression of TLR2 and TLR4 protein and mRNA in human monocytes in a time- and dose-dependent manner (Sadeghi *et al.*, 2006). Vitamin D3 was found to be the only agent that induce CD14 expression among those which induce phenotypic alterations in the THP-1 cells, such as phorbol myristate acetate (PMA) (Fleit and Kobasiuk, 1991; Zhang *et al.*, 1994), It has also been found that Vitamin D3 induced CD14 expression on human monocytes (Sadeghi *et al.*, 2006). Nevertheless, this unexpectedly resulted in impaired TNF-α response, emphasizing the vital role of TLR in the stimulation of inflammation (Sadeghi *et al.*, 2006). Another study found that stimulation of THP-1 cells with Vitamin D3 did not induce a release of TNF-α and challenging both Vitamin D3-differentiated THP-1 cells and PMA-differentiated THP-1 cells with LPS resulted in much lower release of TNF-α from the former comparing with the latter, ~500pg/10^6 and 7000pg/10^6 respectively (Schwende *et al.*, 1996). On the other hand, measuring cytokines was done either by L929 cytotoxicity bioassay for TNF-α or by ELISA for TNF-α and IL-1β. ELISA is favoured by many investigators as it determines total TNF-α produced. While the L929 bioassay measures only biological activity and does not detect receptor-bound TNF-α (Hutchison *et al.*, 2000). Despite the differences between these two methods in their ways of being sensitive, the results demonstrated comparable styles.

4.3 Effects of interaction between different LPSs from different bacteria and extracted by different methods

Four different Gram-negative species were chosen to represent LPSs with different characteristics; *E.coli* as an ideal example of highly active enterobacterial LPS, *B. fragilis* LPS as an example of low active LPS, *Ps. aeruginosa* as a member of non-gut flora and *R. sphaeroides* LPS as a non toxic LPS. Investigation of the interaction between different LPSs is an important approach because firstly it shows deeper insight into the effect of endotoxins against host cells and the mechanism of endotoxin bioactivity, and secondly it may lead to new strategies to control endotoxin effects pharmacologically at a very early step (Rietschel *et al.*, 1994).

Choosing 100ng/ml to be the potential concentration for antagonism activity is based on the fact that there is a considerable difference in the endotoxic activity between highly active *E.coli* and low active *B. fragilis* LPSs. So it was useful to pick one concentration which is reasonably high enough for the low active LPS and rational for the high active LPS. Moreover, previous study showed that antagonistic effects were observed when LPSs from both *E. coli* and *P. gingivalis* were each used at 100 ng/ml (Hajishengallis *et al.*, 2002a). Choosing *B. fragilis* in particular to elucidate the potential antagonism activity of their LPS against the classical kind of LPS such as *E. coli* was a fundamental approach of this study. In general, *Bacteriodes* spp far outnumber all other bacterial flora and account for 20-30% of the isolated species in the gastrointestinal tract (Maier *et al.*, 1972; Wannemuehler *et al.*, 1984; Patrick, 1993; Neish, 2002). Particularly, it is estimated that *Bacteroides* species outnumber the enterobacterial by 1,000-fold (Poxton and Edmond, 1995). Among anaerobic species, *B. fragilis* represents about 1% of the bacterial flora in intestines (Moore and Holdeman, 1974; Holdeman *et al.*, 1976). Importantly, *B. fragilis* is found as a major component of the mucosa-associated flora (Poxton *et al.*, 1997; Ferreira *et al.*, 2002). It is also of utmost importance as an opportunistic pathogen since it is commonly associated with bacteraemia, soft tissue infections, intra-abdominal infections and abscesses (Gorbach and Bartlett, 1974; Polk and Kasper, 1977; Simon and Gorbach, 1984; Goldstein and Citron, 1988; Finegold, 1995; Redondo *et al.*, 1995; Farthmann and Schoffel, 1998; Brook and Frazier, 2000; Nguyen *et al.*, 2000; Javaloyas *et al.*, 2002). Moreover, it is found that selective gut decontamination was unsuccessful in decreasing the emergence of SIRS, to a certain extent because it just reduced aerobic endotoxin sources but not *B. fragilis* a key contributor to endotoxaemia in the case of translocation of the gut flora (Bennett-Guerrero *et al.*, 2000; Bouter *et al.*, 2002).

The findings of this study regarding the ability of *B. fragilis* LPS to induce proinflammotory cytokines (Fig 3.7) are in keeping with previous results from others who showed that, compared to *E.coli* and some other Gram-negative bacteria, *B. fragilis* has a lower systemic endotoxicity (Sveen *et al.*, 1977; O'Donnell *et al.*, 1980; Simon *et al.*, 1985; Magnuson *et al.*, 1989; Lindberg *et al.*, 1990). LPS of *B. fragilis* has structural differences which may contribute to its lower bioactivity when it is compared with that of enterobacterial LPS (Hofstad *et al.*, 1977; Weintraub *et al.*,

1989; Erridge *et al.*, 2002). However, another study indicated that different extraction methods produce different *B. fragilis* LPSs in term of chemical composition and biological activity (Poxton and Edmond, 1995). Nevertheless, the differences in cytokine response between different *B. fragilis* LPSs, on the other hand, emphasize the notion in the beginning of this chapter that different extraction method produce different LPSs in term of chemical composition and biological activity. The findings of the lowering effect exerted by *B. fragilis* LPSs against *E. coli* LPSs are also consistent with that of Delahooke and others (1995). They found that *B. fragilis* NCTC 9343 LPS at higher concentration can block the effects of *E. coli* LPS on both human PMBC and VD3treated/untreated THP-1 cells (Delahooke *et al.*, 1995). Although it is acknowledged that the results obtained (Sections 3.4 and 3.5, previous chapter) are not of close relationship, Magnuson and others (1989) found that *B. fragilis* LPS can inhibit the induction of endothelial adhesiveness by *E. coli* LPS in a way that the more *B. fragilis* LPS concentration contribute to the more increasing of the inhibition (Magnuson *et al.*, 1989). Moreover, it was also shown that partial deacylation (dLPS) inhibits the ability of LPS, but not other ligands, to stimulate adhesion of neutrophils to human endothelial cells (Pohlman *et al.*, 1987). Like macrophages, vascular endothelial cell are consider to be a critical target for LPS action (Morrison and Ryan, 1987; Cybulsky *et al.*, 1988; Pober and Cotran, 1990; Heumann and Glauser, 1994; Mantovani *et al.*, 1997).

In fact, the chemical and biological properties of *B. fragilis* LPS and its Lipid A are identical to those of LPS from *P. gingivalis,* but different from those of classical enterobacterial LPS such as *E. coli* (Weintraub *et al.*, 1989; Hamada *et al.*, 1990). *B. fragilis* Lipid A is similar to that of *P. gingivalis* in many ways (Ogawa, 1993; Kumada *et al.*, 1995). They both lack phosphorus at position 4 of the disaccharide domain and have five fatty acids chains of 15 to 17 carbon residues. However, the position of the various fatty acids is different (Erridge *et al.*, 2002). Moreover, it is generally accepted that the toxicity of penta-acyl Lipid A is lower than that of hexa-acyl Lipid A (Qureshi *et al.*, 1991). Once again this points out the importance of the notion that although different Lipid A components have an overall similar structures, specific variations contribute to their different activities. Thus, the Lipid A of *B. fragilis* LPS differs from that of *E. coli* in several aspects. 1) The fatty acids of *B.*

fragilis Lipid A have chain lengths of 15 to 17 carbon atoms, whereas those of *E. coli* have 12 to 14 carbons. This is noteworthy since it was shown that biologically more active LPS usually contains shorter fatty acids with 10-14 carbon atoms. 2) There are 4 to 5 fatty acids per diglucosamine residue in *B. fragilis* Lipid A, rather than 6 in *E. coli.* In particular, *B. fragilis* NCTC 9343 Lipid A structure has a considerable heterogenicity in this manner since it contains both tetra- and penta-acyl chains. 3) There is only one β-hydroxy fatty acid substituted with a nonhydroxylated fatty acid in *B. fragilis*, whereas there are two in *E. coli.* 4) *B. fragilis* Lipid A lacks a phosphate domain on the C4 of the non-reducing glucosamine but it has a phosphate group on C1 of the reducing amino sugar. 5) The KDO domain in *B. fragilis* is most likely substituted by a phosphate group(s) not present on *E. coli* KDO groups (Weintraub *et al.*, 1989; Lindberg *et al.*, 1990; Patrick, 1993).

It is interesting to note that the same distribution of five fatty acids in *B. fragilis* Lipid A are also found in the endotoxically non-active Lipid A of *R. sphaeroides* (Weintraub et al., 1989). It is also found that tetraacyl structures of lipopolysaccharide (LPS) and Lipid A act like LPS that has been partially enzymatically deacylated (dLPS) by acyloxyacyl hydrolase. These compounds antagonise other LPS structure such as hexa-acylated LPS at the surfaces of different human cells such as monocyte, neutrophils, endothelial cells and whole blood human cells (Loppnow *et al.*, 1989; Kovach *et al.*, 1990; Nogare and Yarbrough, 1990; Riedo *et al.*, 1990; Golenbock *et al.*, 1991; Lynn *et al.*, 1991; Feist *et al.*, 1992; Kitchens *et al.*, 1992). Kitchens and Munford (1995) demonstrated that such dLPS can antagonize LPS in three ways. 1) When the concentration of LBP in the medium was suboptimal for stimulating LPS-CD14 binding, low concentrations of dLPS were capable of competing with LPS for binding to LBP and inhibit the binding of LPS to CD14 competitively. 2) When LBP was present in excessive concentration, dLPS could compete with LPS for binding CD14, but only at dLPS concentrations that were at or above 100 ng/ml. 3) In contrast, low concentrations of dLPS (1ng/ml) inhibited LPS, at 3ng/ml, induction of interleukin-8 response without antagonizing LPS binding to CD14. Underacylated LPS, such as penta- or tetra- acylated LPSs, have been reported to have an antagonism activity against hexa-acylated LPS like

those of *E.coli*. Penta-acylated LPS isolated from *R. sphaeroides* displays the ability to antagonize *E. coli* LPS (Golenbock *et al.*, 1991). Mainly, tetra-acylated LPS of *P. gingivalis* and the penta-acylated one also have such an antagonism property (Bainbridge *et al.*, 2002; Darveau *et al.*, 2004b). Penta-acylated LPS of *B. fragilis* has also this ability to antagonize *E.coli* LPS (Delahooke *et al.*, 1995). A penta-acylated form of mutant *E. coli* LPS can successfully antagonize the capability of hexa-acylated *E. coli* LPS to activate human endothelial cells (Somerville *et al.*, 1996). In addition, the synthetic penta-acylated Lipid A-like compound, E5531, is also considered as a potential therapeutic antagonist agent for LPS-dependent cell activation and therefore stops septic shock development (Christ *et al.*, 1995; Kawata *et al.*, 1999).

Fig 3.12 demonstrates that *R. sphaeroides* LPS is comparable to the cell culture medium in producing almost no cytokine. This finding is consistent with a previous study showing that when using RAW 264.7 cells and L929 cells as an indicator cell, Lipid A from *R. sphaeroides* was not able to induce TNF-α (Takayama *et al.*, 1989). Moreover, another previous study reported that the LPS of *R. sphaeroides* ATCC 17023 is nontoxic (Strittmatter *et al.*, 1983). Fig 3.12 also shows that *R. sphaeroides* LPS managed to inhibit the effect of *E. coli* and *Ps. aeruginosa* LPSs (A and C). However, there was no measurable differences between the TNF-α produced by different *B. fragilis* LPSs by themselves and *B. fragilis* LPSs with 100μg/ml of *R. sphaeroides* LPS. This may indicate that *R. sphaeroides* LPS has no effective antagonism activity on *B. fragilis* LPSs. In fact, a previous study demonstrated that LPS from *R. sphaeroides* had no inhibition effect on cytokine responses induced by repurified *P. gingivalis,* which has LPS structure identical to that of *B. fragilis,* or *Prevotella intermedia* (Kirikae et al., 1999).

4.4 TLR specificity of *B. fragilis* and *R. sphaeroides* LPSs and heat killed *B. fragilis*

Another main aim of this study was to elucidate the TLR specificity of different *B. fragilis* LPS preparations extracted by different methods and different heat killed *B. fragilis* populations as demonstrated in section 3.7. Although, TLR4, in particular, is considered to be the main transducer of classic LPS activity (Poltorak *et al.*, 1998a;

Hoshino *et al.*, 1999; Hirschfeld *et al.*, 2000; Lien *et al.*, 2000), there are contradictory reports as to whether TLR2 (Erridge *et al.*, 2004a; Erridge *et al.*, 2007a) or TLR4 (Mancuso *et al.*, 2005) play a principal role in signalling of *B. fragilis* LPS, as a non-classical lipopolysaccharide. A recent study has demonstrated that penta-acylated and tetra-acylated LPSs like those of *B. fragilis* and *P. gingivalis* functionally antagonize hexa-acylated LPS in its signalling via human TLR4 by direct binding to the co-receptor human MD2. It has been shown that these antagonistic LPSs can either directly compete with hexa-acylated LPSs, such as *E. coli* LPS, for the same binding site on MD-2; or antagonistic LPS/MD2 complexes can inhibit *E. coli* LPS/MD2 complexes signalling at TLR4 (Coats *et al.*, 2007).

Comparison between Fig 3.30 and Fig 3.31 clearly demonstrated that the repurification method made a huge difference in the intensity of the TLR signalling but contributed nothing to the TLR specificity. The contribution of the repurification method was clearly profound with all extraction methods and especially with TMP and PCP methods. It is also possible to assume that although different extraction methods may affect the conformational properties of Lipid A moieties of different *B. fragilis* LPSs, this effect did not change the TLR specificity. That is the possible reason why all 10 unpurified and purified *B. fragilis* LPSs, together with different heat killed *B. fragilis* populations had an obvious TLR2 specificity through transfection experiment. None of them produced an LPS structure that signalled through TLR4 even after applying the repurification method (see Fig 3.30 and Fig 3.31). Using intact cells of *B. fragilis* in terms of heat killed bacteria of different capsular polysaccharide populations was a further confirmation that *B. fragilis* does not have a ligand that can signal through TLR4 although the intensity of TLR2 specificity by heat-killed *B. fragilis* (Fig 3.32) are comparable to their levels with the unpurified *B. fragilis* LPSs demonstrated in Fig 3.30. Fig 3.32 showed that *R. sphaeroides* LPS also has a notable TLR2 specificity. Probably this is due to the presence of trace amounts of protein contaminants or lipoprotein even after the repurification method. This is obvious when the TLR2 specificity of this LPS, which is lower, is compared with purified RS1p than unpurified RS1u (Fig 3.32).

The method of repurification of LPS preparations to eliminate the protein contaminants has shown notable differences in signalling through either TLR2 or

TLR4. Recent studies have raised the issue of the diversity of these proteins and indicate the need for investigations to clarify that a specific LPS preparation is free from specific protein contaminants. These outer membrane proteins (OMP) include molecules such as murein lipoprotein (MLP), peptidoglycan-associated lipoprotein (PAL), and outer membrane protein A (OmpA) that are found to be present in LPS from rough and smooth bacteria (Hellman *et al.*, 2003). One relevant study also identified two lipoproteins, Lip19 and Lip12 in LPS derived from *Escherichia coli* K-12 strain LCD25 to be responsible for TLR2 signalling (Lee *et al.*, 2002). Moreover, one recent study revealed that PAL is biologically active and is released into serum in close association with LPS. PAL alone or through synergistic action with LPS can induce inflammation via TLR2 (Liang *et al.*, 2005). Furthermore, another study showed that PAL is released into the blood circulation in Gram-negative sepsis and caused an inflammatory immune response that led to death in mice (Hellman *et al.*, 2002). Such findings are in a way supportive to our findings since the TLR2 signalling through heat killed bacteria or unpurified *B. fragilis* LPSs are higher than that of repurified LPSs. This may be in part due to other proinflammatory OMP molecules without excluding the role of ligands such as peptidoglycan which is known to signal via TLR2. Many experimental differences between in vitro assays and in vivo human LPS models should be taken into consideration when trying to understand what really happens inside the host. For example, the in-vitro assay whatever its type, a fresh blood sample from volunteers or cell line culture, are not subjected to the remarkable decrease in numbers, as occurs in in-vivo model, of circulatory monocytes in particular and white blood cells counts in general after LPS administration as shown by Richardson and others (1989). One recent report gave such a difference in another dimension when it was suggested that TLR2 and TLR4 are initially down-regulated on monocytes 2h after the LPS in-vivo administration, and they were then up-regulated reaching a significant level for TLR2 by 8 h (Marsik *et al.*, 2003).

The reason for the inconsistency between this study's findings and Erridge and others (2004a; 2007a) which all showed TLR2 specificity of *B. fragilis* LPS and heat killed *B. fragilis* and those of Mancuso and his colleagues (2005) which showed TLR4 specificity of *B. fragilis* LPS is unclear. However, this discrepancy may be due

to culture conditions or even mixed bacterial cultures. In this regard, the findings of the current study are preferred since it contains a diverse range of *B. fragilis* LPS preparations together with different heat killed whole *B. fragilis* cells.

Although the TLR specificity of the classical LPS, such as those of *E. coli,* went through a period of some controversy, nowadays it is general accepted that TLR4 is an essential receptor for such a kind of LPS despite the fact that as yet there is no clear evidence for the direct physical contact between TLR4 and LPS. This is not to say, however, that TLR4 is the universal and the only receptor from the TLR receptor family that detects LPS structures. In fact the majority of the studies demonstrating TLR4 as an essential receptor for LPS signalling, have concluded this by using enterobacterial LPS of a classical kind especially that extracted from *E. coli* or *Salmonella* (see Table 1.1). This may have occurred because endotoxin preparations from these bacteria are commercially available and the fact that the structures of both are well characterised as mentioned by Erridge and others (2007a). Accordingly, it seems inaccurate to conclude that TLR4-deficient mice do not respond to LPS. It is more precise to say that they do not respond to the effect of classical LPS such as those extracted from *E. coli.* Although a recent study by Mancuso and others (2005) reported that repurified *B. fragilis* LPS failed to induce TNF-α production in macrophages from LPS non-responder C3H/HeJ mice, which have a point mutation in TLR4 that functions as a dominant-negative mutation. The same preparation caused the release TNF-α from peritoneal macrophages from TLR2-deficient mice. But this is not directly evident since there remains the strong possibility that different species of animals use different TLR receptors in LPS signalling as proposed by Takeuchi and others (1999). In fact, one of major disadvantage of using a cell line from a dominant-negative TLR4 mouse strain is the fact that binding of LPS to TLR4 is still taking place at normal levels. Since the mutation point His712Pro is located in the intracellular domain of TLR4, this most likely leaves the LPS binding properties of TLR4 unchanged (Lorenz *et al.*, 2002b). On the other hand, LPS non-responder C57BL/10ScNCr mice, which have a complete deletion of the TLR4 gene, has the ability to produce the cytokine of macrophage inflammatory protein-2 in the absence of TLR4 (Lorenz *et al.*, 2002b). Another study showed that mouse peritoneal macrophages appear not to be the key

cells responsible for the overall host response during endotoxic shock. In fact, it was suggested that the structural requirements for cells like human monocytes to recognize LPS structures are more strict than those for murine cells (Matsuura *et al.*, 1999). Nevertheless, human cells broadly recognize more LPS-antagonist structures compared to those recognized by murine cells (Matsuura *et al.*, 1999). Such findings emphasize the need to validate the association between in-vivo systems and in-vitro systems when attributing specific functions to a cell type (Amura *et al.*, 1998). In the same context, it is found that *P. gingivalis* LPS, which is structurally related to *B. fragilis* LPS, can act on TLR4 in a species-dependent manner since it does not signal through mouse-TLR4 (Hirschfeld *et al.*, 2001) or human-TLR4 (Martin *et al.*, 2001; Hajishengallis *et al.*, 2002c) but shows the ability to signal a hamster-TLR4 and to be an antagonist for human-TLR4 (Yoshimura *et al.*, 2002). Another study found that the same species-dependency for *R. sphaeroides* LPS which has the ability to act as an antagonist for human and mouse TLR4 but as an agonist for hamster TLR4 (Lien *et al.*, 2000). It is probable that murine TLR4 is unable to distinguish between conical and cylindrical conformation styles of Lipid A but human TLR4 is able to do so. In fact a previous study reported that a molecule like Lipid IVa acts as an antagonist for human MD-2, the essential co-receptor for TLR4, but acts as an agonist with murine MD-2 (Akashi *et al.*, 2001). The possible reason for the difference between human and mouse TLR4 is probably the differences between mouse and human microbial flora in a way the human immune system needs another receptor to differentiate between "danger" and "non-danger" LPSs but the mouse immune system does not need such an extra receptor since it considers all LPS species as a "danger" signal.

It has been reported that *P. gingivalis* LPS, which was used as a TLR2 positive control in this study, is highly heterogeneous since it contains more than one Lipid A structure. This is why it is sometimes considered as a natural antagonist for *E. coli* LPS and other oral bacteria and in term of TLR specificity it is also reported to be a TLR4 antagonist in some cell types (Darveau *et al.*, 2004b). However, *P. gingivalis* LPS extracted from lipoprotein-deficient mutant showed a noticeable decline in TLR2 signalling (Asai *et al.*, 2005). Moreover, another study showed that underacylated LPSs such as tetra- or penta-acylated LPSs produce reduced TLR4-

dependent cell stimulation by shifting the interaction of the LPS/MD-2 complex with TLR4 in a way that lessens receptor activation (Teghanemt *et al.*, 2005).

Thus it could be hypothesized that different LPS species signal through different TLR receptors. In fact it is tempting to speculate that TLR4 seems to be the essential receptor for the "danger" LPS while other kinds of LPS which are known to be less active than classical LPS in their ability to induce immune responses seem to be detected via other member of TLR family such as TLR2. This may be conceivable in the light of a large body of evidence that addresses the broad specificity of TLR2 as a receptor for a wide range of bacterial structures which are considered to be low immune inducers compared with LPS from *E. coli* for example. Furthermore, many studies observed that TLR2-transfected cells respond to a wide range of bacteria and bacterial products, including Gram-positive bacteria, spirochaetes, mycoplasma and mycobacteria (Kirschning *et al.*, 1998; Yang *et al.*, 1998; Brightbill *et al.*, 1999; Hirschfeld *et al.*, 1999; Lien *et al.*, 1999; Means *et al.*, 1999a; Means *et al.*, 1999b; Schwandner *et al.*, 1999; Yoshimura *et al.*, 1999). This situation makes it difficult to identify a common microbial pattern among all the ligands that are detected by a single TLR2 molecule. Nevertheless, there is no clear evidence that TLR2 directly binds any of these ligands (Lien *et al.*, 1999). One study indicated that TLR2 tends to form a functional complexes with TLR6 in order to increase its range of recognition (Ozinsky *et al.*, 2000). This complex formation may occur between either TLR2 and other TLRs or other TLRs with each other.

From the genetic point of view, there is no reason to suppose that *Tlr4* is the only gene that makes up the core of signalling machinery since one recent study has reported the presence of new locus, *Lps2*, which is required for TNF production in response to LPS (Hoebe *et al.*, 2003b). The *Lps2* locus is similar in phenotypic effect to *Tlr4* and does not represent a novel allele of any of the genes that are known to determine LPS signalling mechanism. Unlike *Tlr4*, the *Lps2* locus does not exclude signalling initiated by peptidoglycan or unmethylated DNA (Hoebe *et al.*, 2003a; Hoebe *et al.*, 2003b).

Moreover, new players have come on the scene and are currently attracting a growing level of attention. This is a group of molecules that show a negative

regulation activity against TLRs and include SIGIRR, IRAK-M, MyD88s, Tollip, ST2, Nod2 and Triad3A (Kobayashi *et al.*, 2002; Zhang and Ghosh, 2002; Burns *et al.*, 2003; Wald *et al.*, 2003; Chuang and Ulevitch, 2004; Watanabe *et al.*, 2004). Of much interest is RP105 which was originally considered as a B cell specific molecule capable of controlling B cell proliferation (Miyake *et al.*, 1994; Miyake *et al.*, 1995). One recent study has shown that RP105 is a specific homologue of TLR4 and is not only found on B cells. Moreover, a complex of RP105/MD-1 directly interacts with TLR4/MD-2 to inhibit its ability to bind LPS in a dose-dependent manner in a HEK293 cells assay. Compared to wild-type mice, RP105-deficient mice produced notably more TNF-α in response to a low dose of *E.coli* LPS while a high dose of the same LPS stimulated an augmented and accelerated endotoxicity. This strongly implicates RP105 as a physiological negative regulator of TLR4 responses (Divanovic *et al.*, 2005). Such findings add another level of competition to LPS signalling since it raises the possibility of negative regulation molecules for other TLRs or even multiple negative regulatory activities for a molecule such as RP105.

4.5 Proposed scenario for non-classical LPS community in the gut and their signalling receptor

The intention of this section is to try to summarize our understanding on two fronts, the role of gut flora as the main source of endotoxin in septicaemia and the role of TLR2 as a potential receptor for non-classical LPS. Innate immune mechanisms, such as epithelial production of α-defensins, mucins and secretory immunoglobulin A contribute to the prevention of bacteria from crossing the mucosal barrier (Hooper and Gordon, 2001). Moreover, non-pathogenic bacteria may directly enhance the capacity of intestinal epithelium to limit the immune response. In fact, one study showed that an avirulent *Salmonella* strain abolished the induction of inflammatory cytokines in human intestinal epithelial cells by interacting with human epithelia to inhibit the NF-κB activation pathway by stopping IκB-α degradation, which eventually prevents subsequent activation of NF-κB dimmer (Neish *et al.*, 2000). Additionally, commensals like *Bacteroides thetaiotaomicron* are found to antagonise transcription factor NF-κB selectively (Kelly *et al.*, 2004). These findings suggest a

kind of tolerance mechanism is involved by the commensals that protect against potential proinflammatory stimuli (Neish *et al.*, 2000). Furthermore, the presence of anaerobic bacteria prevent the enteric colonization of exogenous bacteria via "colonization resistance" processes (van der Waaij *et al.*, 1972). These bacteria also appeared to play a role in preventing the translocation of indigenous gut bacteria. One previous study showed that the administration of metronidazole, which selectively eliminates gut anaerobic bacteria, to mice significantly contributed to increased rates of dissemination of intestinal bacteria into mesenteric lymph nodes. This kind of bacterial dissemination did not occur when mice were pretreated with streptomycin, which selectively eliminate gut facultative Gram-negative bacilli. This finding suggests that the absence of anaerobic bacteria facilitated the translocation of the intestinal facultative anaerobic bacteria (Wells *et al.*, 1987). This may explain the observation that facultative anaerobic gut flora are the most common translocated organism that cross the intestinal barrier in cases of sepsis when these ecological/immunological barriers are no longer effective. In fact, one previous study reported that *E. coli* was the translocated organism identified in 54% of cases (O'Boyle *et al.*, 1998)

In the gut environment, intestinal epithelial cells are considered as an essential barrier that prevent the translocation of the gut flora or its products to the blood circulation. These cells are non-responsive to LPS and other bacterial products since they either express low levels or do not express CD14, TLR2 and TLR4 molecules and the co-receptor MD-2 (Cario and Podolsky, 2000; Abreu *et al.*, 2001; Melmed *et al.*, 2003; Fischer *et al.*, 2006). However, another study showed that TLR2 mRNA was detected in human intestinal epithelium whereas TLR4 mRNA was not (Naik *et al.*, 2001b). At mucosal surfaces, epithelial cells have the capacity to respond to pathogens by secreting chemokines that recruit circulating polymorphonuclear cells and monocytes (Kagnoff and Eckmann, 1997; Mahida and Johal, 2001). Such findings seem to be scientifically logical as the intestinal epithelium should stay immunologically inert in response to gut flora and their LPS, but must retain its capability to express TLR4 when "danger" LPS (i.e. TLR4 agonist) is sensed (Abreu *et al.*, 2002)

Other potential factors came from one recent study which has demonstrated that although TLR2 surface expression is unaffected by aging, it has been shown that there was a highly significant defect in TLR1/2-induced TNF-α and IL-6 production from peripheral blood monocytes of older adults (>65 years) compared with young controls (21–30 years) (van Duin *et al.*, 2007). This finding may shed some light on findings from another study that showed that microbial translocation of gut origin flora significantly increases with elderly patient (>70 years) (O'Boyle *et al.*, 1998). On the other hand, monocyte mRNA and cell-surface receptor expression of TLR4 were increased 2.4-fold ($P < 0.05$) versus 1.7-fold ($P < 0.02$) in patients compared with normal controls respectively(Calvano *et al.*, 2003).

In the light of these findings, one can propose a scenario relating to what happens in the gut when endotoxin is translocated to the blood circulation to start the events of sepsis. In this case, it has been proposed that *B. fragilis* LPS has a protective role if it is assumed as "non-danger" LPS which may inhibit classical kind of LPS which is proposed to be kind of "danger" LPS. On the other hand, the ability of *B. fragilis* LPS to induce low cytokine production such as TNF-α, is something that might keep the immune response alert or might contribute to the immune tolerance against LPS. This protective role might be dramatically changed in the case of disruption of the permeability barrier of the large bowel by means of inflammation-mediated mechanisms. Huge amounts of LPS from the gut flora, with anaerobic bacteria representing the main species, may translocate to the blood circulation leading to the probable over stimulation of TLR2 which may contribute to septic shock with other kinds of LPS acting as TLR4-agonists. In fact, *B. fragilis* has the antigenic variation capacity that may contribute to both its proposed protective role as a member of the normal intestinal flora and its pathogenic mode as an opportunistic pathogen (Patrick *et al.*, 1999; Sears, 2005).

Another potential way to evaluate the role of TLR2 in signalling the so called "non-danger" LPS came from a study reporting that blood cells from farmers' children express significantly higher amounts of CD14 and TLR2 on their surfaces than those from non-farmers' children (Lauener *et al.*, 2002). These findings suggested that TLR2 with specificity towards "non-danger" ligands is also affected by the environment. It is proposed that the farm environment contains higher concentrations

of the bacterial cell wall component lipopolysaccharide (endotoxin) than those of non-farming environments. This environmental situation which exerts a relatively high "endotoxic pressure" on the immune system might change some "danger" LPS to be "non-danger" in term of how the immune system deals with it. The more simple interpretation is that TLR2 plays a central role in LPS tolerance dependent on both the kind of LPS and quantity of it. Actually, in-vivo studies on intestinal epithelial cell lines have revealed that extended exposure to LPS or lipoteichoic acid leads to both tolerance and cross-tolerance to other ligands by mechanism which include a decrease in TLR surface expression (Abreu *et al.*, 2005)

4.6 Future directions

There is still much to be addressed in the field of LPS signalling and interactions between different kinds of LPS. Based on the findings from this study, many questions have been brought up that, if answered, would shed more light on the activity of non-classical LPS preparations and its signalling machinery.

1) The interaction between different LPSs that signal different TLR receptor is a direct approach to investigate these kinds of interactions at the TLR level bearing in mind the bacterial species that inhabit the gut use more than one cell system such as human monocytes and intestinal epithelial cells

2) The interactions between different LPSs that propose to have either identical or very similar LPS activities such as different species of *Bacteroides* spp and *P. gingivalis*, or antagonistic LPS activity such as that from *R. sphaeroides*

3) The current findings with others that both TLR4 and TLR2 appeared to be involved in LPS signalling process raise the question as to whether they act independently in a gut environment in particular, or whether they cooperate together to keep the normal balance between "danger" and "non-danger" LPSs in that specific environment.

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